THE NEUROENDOCRINE REGULATION OF JUVENILE HORMONE PRODUCTION IN ADULT FEMALE *HELICOVERPA ARMIGERA* (LEPIDOPTERA:NOCTUIDAE)

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DECLARATION

The research carried out in the course of this investigation and the results presented in this thesis are, except where acknowledged, the original work of the author.

I. J. Rourke

Ian J. Rourke

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ABBREVIATIONS

BE	Brain Extract
CA	Corpus Allatum
CC	Corpus Cardiacum
cDNA	Complementary Deoxyribosenucleic Acid
Dip-AS	*Diploptera punctata* Allatostatin
FA	Farnesoic Acid
FEAR	Fractional Endocrine Activity Ratio
Hea-AT	*Helicoverpa armigera* Allatotropin
HEG CoA	3-Hydroxyl-3-Ethylglutaryl Coenzyme A
HMG CoA	3-Hydroxyl-3-Methylglutaryl Coenzyme A
JH	Juvenile Hormone
LNSC	Lateral Neurosecretory Cell
Mas-AS	*Manduca sexta* Allatostatin
Mas-AT	*Manduca sexta* Allatotropin
MNSC	Medial Neurosecretory Cell
mRNA	Messenger Ribosenucleic Acid
NCA	Nervi Corpori Allati
NCC	Nervi Corpori Cardiaci
NSC	Neurosecretory Cell
PAM	Peptidyl-glycine α-amidating Monooxygenase
TLC	Thin Layer Chromatography

ABSTRACT

This thesis examined several aspects of the neuroendocrine regulation of juvenile hormone (JH) production by the corpus allatum (CA) of adult female *Helicoverpa armigera* (Lepidoptera:Noctuidae). The major emphasis of this work has been the isolation of a gene which encodes a neuropeptide (termed an allatotropin) which stimulates JH synthesis by the *H. armigera* CA.

Juvenile hormone was shown to be essential for oocyte maturation in female *H. armigera*. A radiochemical assay was established to measure the *in vitro* release of JH from isolated CA of this species. JH production was initiated around the time of eclosion, with the rate of JH release increasing over the first day of adult female life. A significant difference in the rates and patterns of JH release was observed for the two strains of *H. armigera* used in this study.

Addition of farnesoic acid (FA) to the radiochemical assay medium greatly increased JH release from the CA of adult female *H. armigera*, but not from the CA of pharate adult moths. This result suggested that one (or both) of the last two enzymes of the JH biosynthetic pathway was either absent or inhibited in the glands of pharate adults and that enzymatic activity appeared rapidly at, or around, the time of eclosion. The experiments with FA also indicated that during adult female life the rate of JH synthesis and release was considerably less than that determined by the activity of the two terminal enzymatic steps.

Chromatographic analyses identified JH III as the predominant homolog released by the CA of adult female *H. armigera in vitro*. Incubation of *H. armigera* CA with the JH precursor FA increased the release of JH III and even more so, JH III diol. JH-degrading esterase activity was found in association with the CA and surrounding tissue.

Brain extracts (BE) prepared from pharate adult and adult *H. armigera,* and synthetic *Manduca sexta* allatotropin (Mas-AT) stimulated JH release from the CA of adult female *H. armigera*. The effects of BE were dose dependant and readily reversible *in vitro*. Neither BE nor Mas-AT preferentially stimulated the production of any specific JH homolog, but JH diol was released in large quantities. A diurnal fluctuation in the sensitivity of the *H. armigera* CA to Mas-AT was observed for the first two days of adult female life, suggesting that the synthesis of JH *in vivo* may be under circadian control.

An antibody generated against the Mas-AT peptide was used to identify sites of Mas-AT expression in brains of female *H. armigera*. The Mas-AT antiserum was immunoreactive with 3 pairs of cells of the sub-esophageal ganglion, 2 pairs of cells

in the lateral region of the tritocerebrum, 6 pairs of cells in the superior lateral region of the protocerebrum, 4 pairs of cells in a posterior lateral region of the protocerebrum and 6-7 pairs of cells in the posterior midline of the brain. Cells responsible for innervation of the CA could not be unambigously determined, but since Mas-AT immunoreactivity was noted in the NCC I/II, the lateral cells of the protocerebrum would be likely candidates. Mas-AT immunoreactivity was found throughout the CA, indicating that the Hea-AT peptide was released from nerve terminals in close proximity to their target cells.

A preliminary study of CA ultrastructure was made on the CA of a newly eclosed adult female *H. armigera*. The cells possessed three features characteristic of glands actively synthesizing JH which have been observed in other species; large and copious intercellular spaces, considerable quantities of mitochondria and extensive layers of smooth endoplasmic reticulum. Numerous nerve endings containing electron dense vesicles were observed in the neurohemal sheath surrounding the CA, and within the CA itself.

Oligonucleotide probes based on the sequence of the Mas-AT peptide were used to isolate the region encoding this peptide from a *M. sexta* genomic DNA library. The Mas-AT clone was then used to isolate the homologous region from a *H. armigera* genomic library, which in turn, was used to obtain a partial cDNA clone encoding the Hea-AT preprohormone from an adult *H. armigera* brain cDNA library.

Alignment of the Hea-AT genomic and Hea-AT cDNA sequences suggested that the Hea-AT gene was composed of at least three exons, A 5' exon(s) that was non-coding, the Hea-AT coding exon which was at least 162bp in length and a 3' exon(s). However, no sequence resembling a splice consensus site was present in the genomic DNA at the point of divergence between the Hea-AT genomic and cDNA clones. Therefore, at present it is uncertain whether the 240 downstream nucleotides of the Hea-AT cDNA clone represents part of the Hea-AT gene.

The 54 N-terminal amino acids of the Hea-AT preprohormone and the 52 N-terminal amino acids of the Mas-AT preprohormone were characterized. A single copy of the allatotropic peptide was contained within these putative preprohormones. The sequence of the biologically active peptide was completely conserved between the two moth species. The putative precursor proteins contained the structural features expected of a preprohormone, including a 20-25 amino acid N-terminal hydrophobic signal sequence and a glycine residue C-terminal to the active peptide which probably acts as a substrate for α-amidation. The allatotropin sequence (plus the C-terminal glycine) was flanked by a single arginine on the N-terminal side and a dibasic, lysine-arginine on the C-terminal side, which could serve as recognition sites for endopeptidase cleavage to liberate the active peptide.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

The neuroendocrine system has been touted as a suggested target for future, biologically rational approaches to insect pest control (Evans *et al.*, 1989; Menn and Borkovec, 1989; Kelly *et al.*, 1990; Masler *et al.*, 1993). In most cases, very little is known about the many neuropeptides which control processes of growth, development and reproduction that might form the basis for such a control strategy. My Ph.D. research has been an investigation of the neuroendocrine control of juvenile hormone (JH) synthesis in adult female *Helicoverpa armigera*, with particular emphasis on the molecular characterization of a gene encoding a neuropeptide involved in this process.

To provide a background to material presented in this thesis an overview of what is known about JH and its neuroendocrine regulation is given below. The first section is a basic introduction to insect endocrinology with emphasis on the role of JH in egg production. The second section deals with JH structure, synthesis and *in vivo* titre regulation. A detailed account of what is already known about the neuroendocrine regulation of JH synthesis in insects is given in the third section. Three species, *Diploptera punctata* (Blattodea), *Locusta migratoria* (Orthoptera) and *Manduca sexta* (Lepidoptera), are discussed in more detail because they are the best characterized with respect to the neuroendocrine regulation of JH synthesis. Common features of neuropeptide preprohormone structure are presented in the fourth section, as these characteristics will be used to identify putative preprohormone genes from DNA sequences isolated from the *H. armigera* genome (Chapter 4). The Australian Cotton Bollworm, *H. armigera* (Lepidoptera:Noctuidae) is a major agricultural pest, particularly of cotton, oilseeds, horticultural and coarse grain crops (Zalucki *et al.*, 1986). A general introduction to the biology of *H. armigera* is provided in the fifth section, followed by a brief outline of the remainder of this dissertation.

1.1 GENERAL INSECT ENDOCRINOLOGY

The essential role of secreted chemical messengers in multicellular organisms is well established. In insects three major classes of such messengers exist; the ecdysteroids, the neurohormones and the juvenile hormones (JH) (Keely and Hayes, 1987). The function of each of these classes is discussed independently, with greatest emphasis on JH.

1.1.1 ECDYSTEROIDS

Ecdysteroid is a generic term used to refer to a family of ecdysone-related steroid hormones. In general they consist of a full C_{27} carbon skeleton of cholesterol, with a *cis* fusion (5β) of the A and B rings, a 6-keto-7-ene system within the B ring and numerous hydroxyl groups (Smith, 1985). In most cases ecdysteroids are synthesized from cholesterol (Warren and Hetru, 1990). In phytophagous insects, such as the Lepidoptera, cholesterol is obtained by dealkylation of phytosterols (Warren and Hetru, 1990). Ecdysteroids are produced throughout insect development predominantly in the prothoracic glands (Chino *et al.*, 1974; Rees, 1985; Smith, 1985) and some secondary sources (Delbecque *et al.*, 1990). Many different cellular targets exist for ecdysteroids, each responding to ecdysteroids in a particular temporal and spatial pattern. These targets can be grouped into three main categories, involving moulting (see section 1.1.3.1), growth and gametogenesis (Koolman, 1990).

1.1.2 NEUROHORMONES

Hundreds of structurally divergent neurohormones are believed to exist in insects. They are generally peptides synthesized by specialized neurons in the nervous system and control specific aspects of growth, development, reproduction and general homeostasis. Their effects extend to roles in the regulation of ecdysteroid and juvenile hormone production (Keeley and Hayes, 1987; Menn and Borkovec, 1989; Holman *et al.*, 1990; Nässel, 1993).

1.1.3 JUVENILE HORMONE

The juvenile hormones are a family of acyclic sesquiterpenoid epoxide compounds (see section 1.2.2) synthesized in the corpus allatum (CA) (see section 1.2.1) at various stages throughout the insect life cycle. The effects of JH are widespread and only the two most comprehensively studied, metamorphosis and oocyte maturation, are discussed below. A summary of the plethora of other known or suspected roles of JH in insect development is provided in Appendix 1.

1.1.3.1 Metamorphosis

There are three types of insect metamorphic strategies.

a) Primitive wingless insects termed Apterygota, where the newly hatched insect grows into an adult without any significant change in the body parts or their proportions,

b) Hemimetabolous insects which go through a number of nymphal stages, punctuated by moulting, with each stage gaining greater resemblance to an adult,

c) Holometabolous insects which go through three postembryonic stages, larval, pupal and adult (Ross, 1965).

In each case the presence of JH maintains juvenile characteristics, preventing adult development (Kumaran, 1990). This function of JH was first alluded to when Wigglesworth (1936) discovered that removal of the head would result in precocious metamorphosis in nymphal *Rhodnius prolixus,* with CA implantation preventing metamorphosis. Following this finding,Bounhiol(1937) induced pupal formation by the removal of the CA in third instar *Bombyx mori* larvae.

The "classical" model for the hormonal control of metamorphosis in holometabolous insects is based on the presence of high levels of JH during a larval/larval moult, low JH levels during a larval/pupal moult and the absence of JH during a pupal/adult moult (Schneiderman and Gilbert, 1964; Gilbert and King, 1973; Richards, 1981). Typically JH titre remains negligible during the pupal stage and is synthesized in the adult for its vitellogenic (see section 1.1.3.2) and other roles (Appendix 1). It should be noted that the complexity of the system greatly exceeds the simplicity of the above model (Jungreis, 1979).

1.1.3.2 Oocyte Maturation

There are many varied strategies for egg production in insects (Engelmann, 1970 and 1983; Bownes, 1986). Apart from a few exceptions in the Diptera, Phasmida and Lepidoptera (Engelmann, 1983; Kumaran, 1990; also see section 1.1.3.2.3), JH has been implicated in egg maturation. As the modes of reproduction vary between species, so do their hormonal regulation, but in general JH titre is high during vitellogenesis and low during previtellogenesis and choriogenesis (Tobe and Stay, 1985).

To understand the neuroendocrine regulation of JH synthesis during adult female development in *D. punctata* (section 1.3.2.1), *L. migratoria* (section 1.3.2.2) and lepidopterans (*M. sexta*; section 1.3.2.4), the reproductive strategies and endocrinology of these insects is presented below.

1.1.3.2.1 *D. punctata*

D. punctata is a viviparous cockroach, in which the eggs mature synchronously. Following mating, egg development takes 10 days, after which the eggs are oviposited in the brood sac. The female undergoes a pregnancy lasting 65-100 days which is terminated by the parturition of larvae, or the laying of eggs close to hatching. Following parturition of the first batch of young, the female can then oviposit the next batch into the brood sac within 7 days (Roth and Stay, 1961; Stay and Coop, 1973; Engelmann, 1970; Tobe, 1980).

One of the strongest correlations between rate of JH synthesis and stage of egg maturation for any species is that observed in *D. punctata.* Upon mating, JH production from the CA increases and is coupled with the period of rapid vitellogenic growth until

the eggs are close to full size. JH synthesis then rapidly declines and remains low until the end of pregnancy, when presumably the second cycle of oocyte development requires the same pattern of JH synthesis (Tobe and Stay, 1977; Tobe, 1980; Johnson *et al.*, 1985; Rankin and Stay, 1985).

1.1.3.2.2 *L. migratoria*

Oocyte development in *L. migratoria* is also synchronous, with the first cycle beginning around eight days after adult emergence and the first batch of eggs being oviposited 8 days later. The second and subsequent batches take less time and egg development starts before the previous batch has been chorionated and oviposited (Ferenz and Kaufner, 1981; Girardie *et al.*, 1981; Feyereisen, 1985b).

Cycles of oocyte development in adult female *L. migratoria* are JH dependent. CA activity has been correlated with vitellogenesis, but this relationship is relatively imprecise, presumably due to the overlapping nature of these cycles (Ferenz and Kaufner, 1981; Girardie *et al.*, 1981; Feyereisen, 1985b; Tobe and Stay, 1985). There is strong evidence that JH regulates synthesis of the vitellogenins as well as their uptake by the developing oocytes in adult female *L. migratoria* (Chen *et al.*, 1976; Wyatt, 1990).

1.1.3.2.3 Lepidoptera

Although numerous different reproductive strategies exist in the Lepidoptera (Engelmann, 1983), the hormonal regulation of egg production broadly falls into two main groups, discussed as group I and group II below. In both types, egg production appears to be continuous and asynchronous. Apparently mating is not necessary for egg laying in some species as virgin *Helicoverpa zea* (Satyanarayana *et al.*, 1991) and *H. armigera* (personal observation) oviposit numerous eggs, however virgin *M. sexta,* at most, only oviposit a few eggs (Sasaki and Riddiford, 1984).

Some species of Lepidoptera have short adult life spans, do not feed as adults and mature their eggs during the pupal stage (group I, Table 1.1). These species require ecdysteroids for the process of egg production, while JH has an inhibitory effect on egg maturation (Pan, 1977; Tsuchida *et al.*, 1987; Davis *et al.*, 1990; Shirk *et al.*, 1990; Hiremath and Jones, 1992). In at least one species, *Galleria mellonella,* JH titres increase after adult female eclosion, with JH probably being required for other processess related to reproduction, such as accessory gland function (Rembold and Sehnal, 1987; also see Appendix 1).

Those Lepidoptera which live longer as feeding adults and mature their oocytes after adult eclosion are referred to as group II in Table 1.1. As with *D. punctata* and *L. migratoria,* JH is essential for egg production in these species. However correlations between vitellogenesis and JH synthesis are impossible to make because of the

SPECIES	FAMILY	REFERENCE
GROUP I		
Bombyx mori	Bombycidae	Tsuchida *et al.*, 1987
Corcyra cephalonica	Pyralidae	Deb and Chakravorty, 1981
Galleria mellonella	Pyralidae	Rembold and Sehnal, 1987
Hyalophora cecropia	Saturniidae	Pan, 1977
Lymantria dispar	Lymantriidae	Davis *et al.*, 1990
Malacosoma pluviale	Lasiocampidae	Sahota, 1969
Plodia interpunctella	Pyralidae	Shirk *et al.*, 1990
Samia cynthia ricini	Saturniidae	Takahashi and Mizobata, 1975
GROUP II		
Agrostis ipsilon	Noctuidae	Gadenne, 1993
Danaus plexippus	Nymphalidae	Pan and Wyatt, 1971
Helicoverpa zea	Noctuidae	Satyanarayana *et al.*, 1991
Heliothis virescens	Noctuidae	Ramaswamy and Cohen, 1991
Leucania separata	Noctuidae	Wu and Quo, 1963
Manduca sexta	Sphingidae	Nijhout and Riddiford, 1974
Nymphalis antiopa	Nymphalidae	Herman and Bennett, 1975
Papilio xuthus	Papilionidae	Fukuda and Kondo, 1965
Pieris brasicae	Pieridae	Karlinsky, 1963
Polygonia c-aurem	Nymphalidae	Endo, 1970
Pseudaletia unipuncta	Noctuidae	Cusson *et al.*, 1990
Spodoptera litura	Noctuidae	Hatakoshi *et al.*, 1992

Table 1.1; Endocrine control of egg maturation in the Lepidoptera. Group I require ecdysteroids, group II JH.

asynchronous nature of oocyte development in these lepidopterans. Fluctuations in JH synthesis have been observed in these adult females, with mating and nutritional state also having a strong influence on JH levels (Nijhout and Riddiford, 1979; Sasaki and Riddiford, 1984; Ishizaka *et al.*, 1987; Cusson *et al.*, 1990; Satyanarayana *et al.*, 1991).

Within the species presented as group II (Table 1.1) there appear to be variations in the exact roles of JH during oocyte maturation (Satyanarayana *et al.*, 1992). In *M. sexta* JH is not needed for the initial period of vitellogenesis which usually occurs late in pupal development, although once the follicles reach 1mm in size (mature size approximately 1.7mm) further maturation is JH dependent (Nijhout and Riddiford, 1979). It appears that JH in this instance is associated with the uptake of yolk proteins (Nijhout and Riddiford, 1979). In contrast, both yolk protein synthesis and uptake are JH dependent in *H. zea* (Satyanarayana *et al.*, 1992).

1.2 JUVENILE HORMONE

1.2.1 CORPUS ALLATUM

The CA is a glandular endocrine organ found in all insects (Cassier, 1990) and in some insects, such as *M. sexta,* it also has a neurohemal function (O'Brien *et al.*, 1988). The gland is usually paired, but in some Hemiptera, Plecoptera and Diptera it is unpaired (Engelmann, 1970; Cassier, 1990). The CA is the only known site of JH biosynthesis in all insects so far studied (Cassier, 1990; Feyereisen, 1985b; Tobe and Stay, 1985; Baker, 1990). Of the insect orders examined, four broad histological types of CA cells have been characterized, termed lymphoid, small, macro and vesicular (Cassier, 1990). Lepidoptera, Trichoptera, Hymenoptera and some Diptera, belong to the macro-cell type, with the gland consisting of a few large cells, which is believed to be the most highly evolved CA morphological type (Cassier, 1990; Tobe and Stay, 1985).

The structure and innervation of the retrocerebral complex, which consists of the CA and corpora cardiaca (CC), varies among insect species (Cassier, 1990; Raabe, 1989) and during development within some species (Copenhaver and Truman, 1986b). A diagram of the brain/retrocerebral complex from adult *M. sexta* is presented in Figure 1.1. The neural connections are of importance in the regulation of both glandular and neurohemal activity of the CA (Tobe and Stay, 1985). In adult *M. sexta* many neurosecretory nerves terminate in the CA, some of these are shown in Figure 1.1. However, for detailed diagrams of axonal ramifications from the brain to the retrocerebral complex in adult *M. sexta,* refer to Copenhaver and Truman (1986b) and Eaton (1988). The large neurosecretory cells identified by Copenhaver and Truman (1986b) (Figure 1.1) terminated in 'blind varicosities within the more superficial regions of the CC and

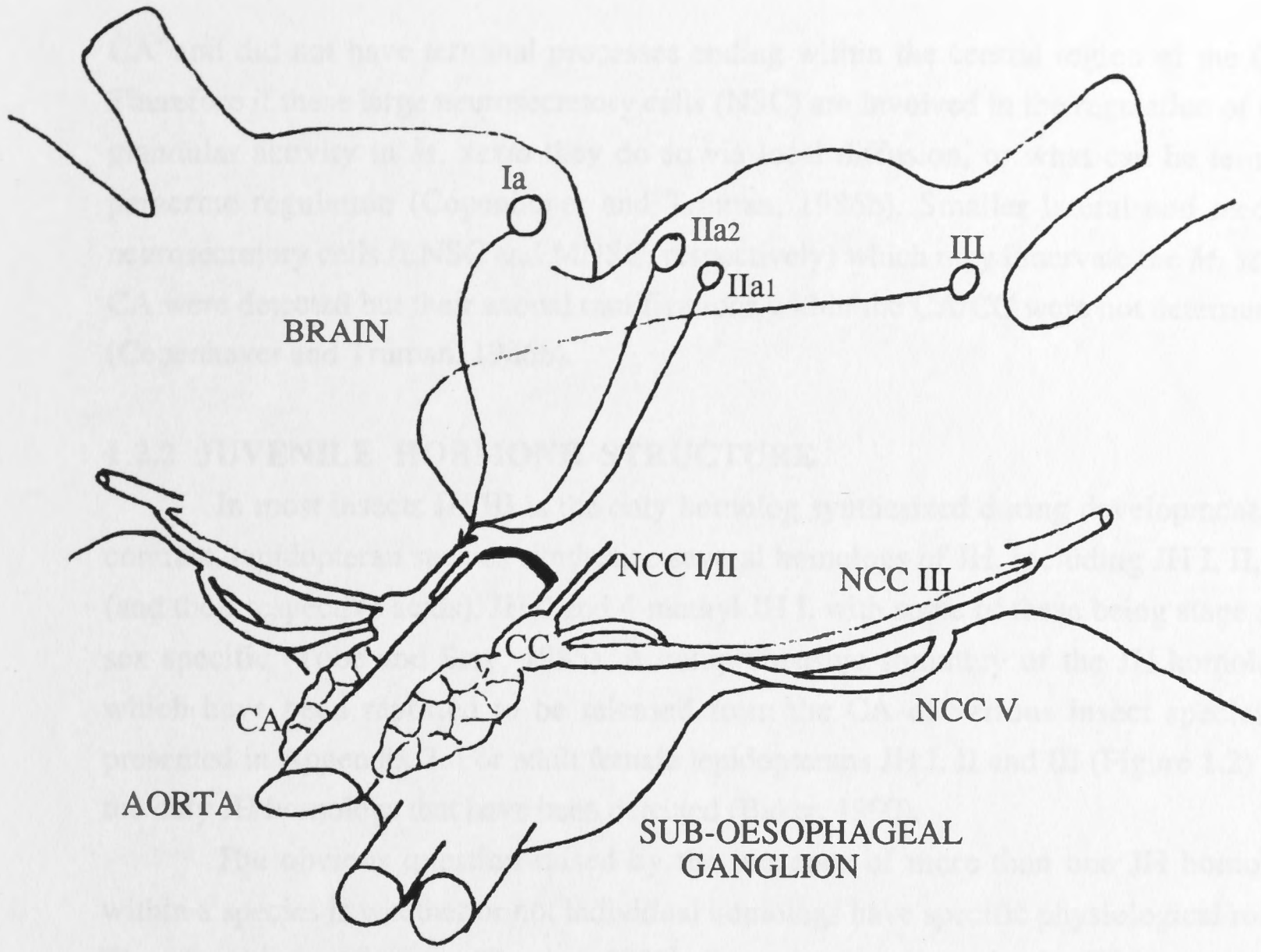


Figure 1.1; Structure of the brain/retrocerebral complex in adult *M. sexta* showing some of the neurosecretory cells (NSC) which innervate the CA (adapted from Copenhaver and Truman, 1986b). Only one set of each bilaterally paired cluster of NSC is shown, represented as open circles. Axons arising from each group of NSC reach the retrocerebral complex via the nervi corpori cardiaci I/II (NCC I/II). Group Ia cells innervate both the ipsilateral CC and CA. Group IIa1 cells project axons only to the contralateral CC, and axons from Group IIa2 and Group III cells terminate in the contralateral CA. However for diagrams of axonal ramifications within the CA refer to Copenhaver and Truman (1986b).

CA' and did not have terminal processes ending within the central region of the CA. Therefore if these large neurosecretory cells (NSC) are involved in the regulation of CA glandular activity in *M. sexta* they do so via local diffusion, or what can be termed paracrine regulation (Copenhaver and Truman, 1986b). Smaller lateral and medial neurosecretory cells (LNSC and MNSC, respectively) which may innervate the *M. sexta* CA were detected but their axonal ramifications within the CA/CC were not determined (Copenhaver and Truman, 1986b).

1.2.2 JUVENILE HORMONE STRUCTURE

In most insects JH III is the only homolog synthesized during development. In contrast, lepidopteran species synthesize several homologs of JH, including JH I, II, III (and their respective acids), JH 0 and 4-methyl JH I, with some of these being stage and sex specific (Tobe and Stay, 1985). A comprehensive summary of the JH homologs which have been reported to be released from the CA of various insect species is presented in Appendix 2. For adult female lepidopterans JH I, II and III (Figure 1.2) are the only JH homologs that have been detected (Baker, 1990).

The obvious question raised by the presence of more than one JH homolog within a species is whether or not individual homologs have specific physiological roles. Thus far this is unknown (Cassier, 1990). Some intermediates in the JH biosynthetic pathway, such as farnesoic acid (FA), methyl farnesoate and the JH acids (see Appendix 2), may be released into the hemolymph as prohormones, with JH synthesis being completed in other tissues (Cusson *et al.*, 1991). This is certainly the case for the JH acids, as the male reproductive tract (Weirich and Culver, 1979; Shirk *et al.*, 1983; Bhaskaran *et al.*, 1988) and the prepupal imaginal discs (Sparagana *et al.*, 1984 and 1985; Bhaskaran *et al.*, 1986) of some Lepidoptera possess JH methyl transferase activity, the final step of JH synthesis in Lepidoptera (Figure 1.3).

During development there are variations in JH homolog ratios in lepidopterans, both within and between species (Granger *et al.*, 1982; Bhaskaran *et al.*, 1986; Ishizaka *et al.*, 1987; Cusson *et al.*, 1990; Satyanarayana *et al.*, 1991; also see Baker, 1990) making assumptions about homolog specific roles difficult. The relative morphogenetic and gonadotropic effects of JH I, II and III have been studied in lepidopteran and non-lepidopteran species, but no clear trends have been noted (Nijhout and Riddiford, 1974; Dahm *et al.*, 1976; Engelmann, 1979; Koeppe *et al.*, 1980; Kelly *et al.*, 1981; Schooley *et al.*, 1984; Ramaswamy and Cohen, 1991). Interestingly in non-lepidopterans, JH I and II apparently possess biological activity equal to or greater than the endogenous hormone, JH III (Koeppe *et al.*, 1980; Kelly *et al.*, 1981). It is clear that considerably more work has to be done to clarify the above issues.

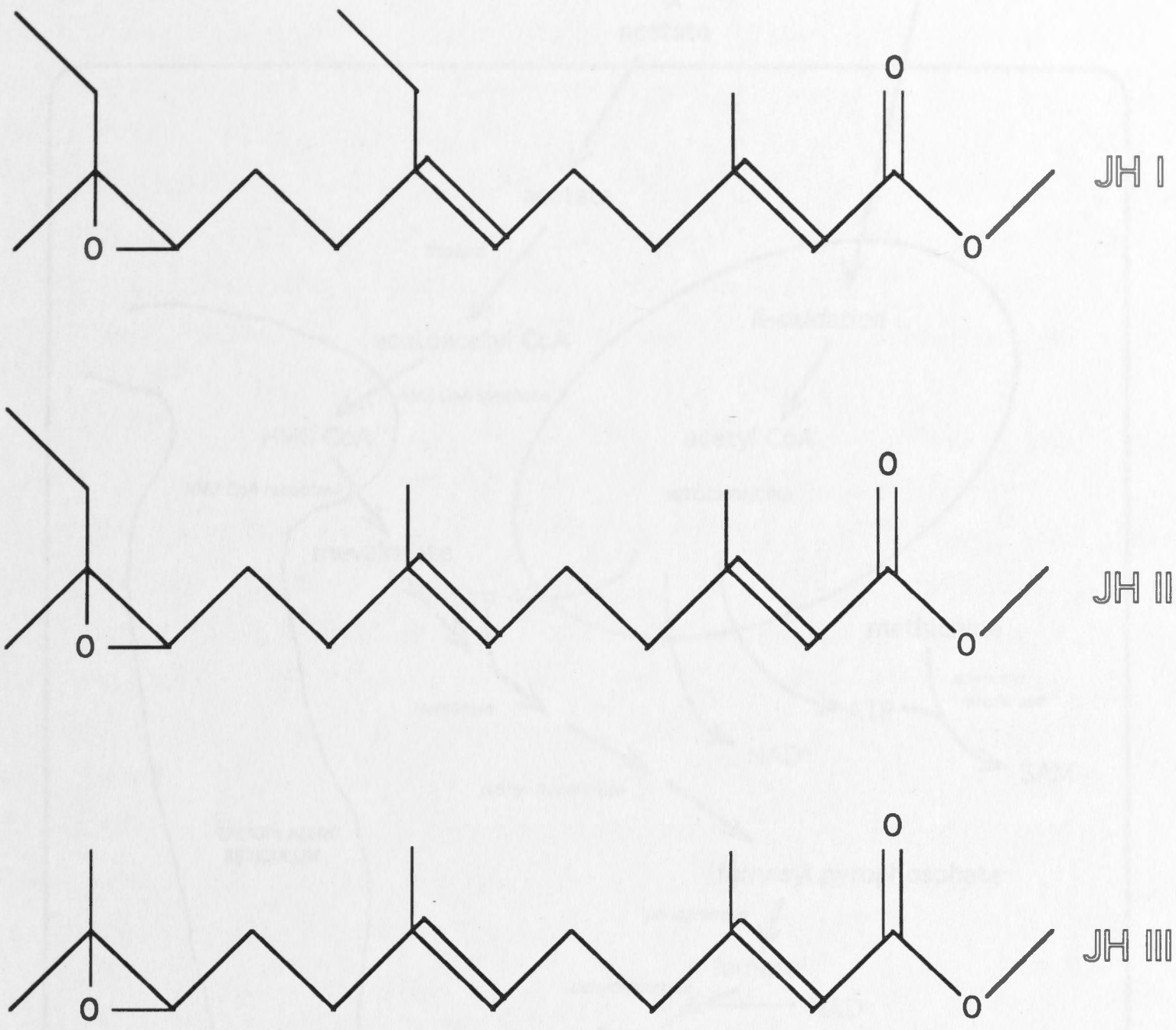


Figure 1.2; Structure of JHs I, II and III, the most common products released from the CA of adult female lepidopterans (Baker, 1990).

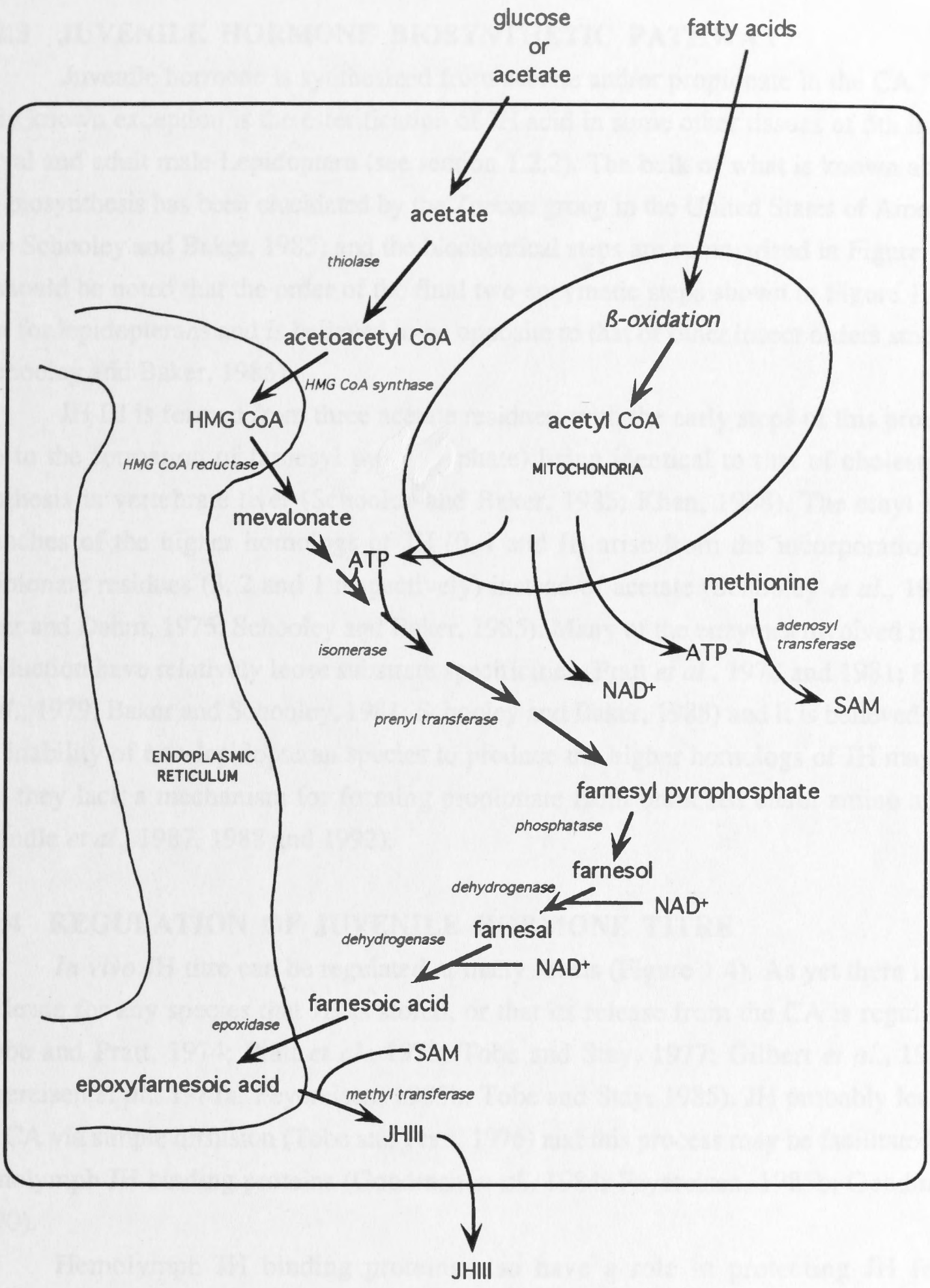


Figure 1.3; Features of the JH III biosynthetic pathway in lepidopterans (adapted from Tobe and Stay, 1985). The conversion of HMG CoA to mevalonate and the conversion of farnesoic acid to epoxyfarnesoic acid occur in the endoplasmic reticulum, with other enzymatic steps taking place in the cytosol. JH I and JH II are derived from the incorporation of two or one (respectively) propionate residues instead of acetate.

1.2.3 JUVENILE HORMONE BIOSYNTHETIC PATHWAY

Juvenile hormone is synthesized from acetate and/or propionate in the CA. The only known exception is the esterification of JH acid in some other tissues of 5th instar larval and adult male Lepidoptera (see section 1.2.2). The bulk of what is known about JH biosynthesis has been elucidated by the Zoecon group in the United States of America (see Schooley and Baker, 1985) and the biochemical steps are summarized in Figure 1.3. It should be noted that the order of the final two enzymatic steps shown in Figure 1.3 is that for lepidopterans and is believed to be opposite to that of other insect orders studied (Schooley and Baker, 1985).

JH III is formed from three acetate residues, with the early steps of this process (up to the formation of farnesyl pyrophosphate) being identical to that of cholesterol synthesis in vertebrate liver (Schooley and Baker, 1985; Khan, 1988). The ethyl side branches of the higher homologs of JH (0, I and II) arise from the incorporation of propionate residues (3, 2 and 1 respectively) instead of acetate (Schooley *et al.*, 1973; Peter and Dahm, 1975; Schooley and Baker, 1985). Many of the enzymes involved in JH production have relatively loose substrate specificities (Pratt *et al.*, 1978 and 1981; Peter *et al.*, 1979; Baker and Schooley, 1981; Schooley and Baker, 1985) and it is believed that the inability of non-lepidopteran species to produce the higher homologs of JH may be that they lack a mechanism for forming propionate from branched chain amino acids (Brindle *et al.*, 1987, 1988 and 1992).

1.2.4 REGULATION OF JUVENILE HORMONE TITRE

In vivo JH titre can be regulated at many levels (Figure 1.4). As yet there is no evidence for any species that JH is stored, or that its release from the CA is regulated (Tobe and Pratt, 1974; Pratt *et al.*, 1975; Tobe and Stay, 1977; Gilbert *et al.*, 1978; Feyereisen *et al.*, 1981a; Feyereisen, 1985b; Tobe and Stay, 1985). JH probably leaves the CA via simple diffusion (Tobe and Pratt, 1976) and this process may be facilitated by hemolymph JH binding proteins (Goodman *et al.*, 1984; Feyereisen, 1985b; Goodman, 1990).

Hemolymph JH binding proteins also have a role in protecting JH from degradative enzymes (see Hammock, 1985; Tobe and Stay, 1985; Roe and Venkatesh, 1990). The two major pathways of JH degradation are ester hydrolysis by JH esterases to form JH acid (Jones and Click, 1987; Venkatesh *et al.*, 1988; Valaitis, 1991) and epoxide hydration by JH epoxide hydrolases which results in JH diol (Wisniewski *et al.*, 1986a,b; Casas *et al.*, 1991; Jesudason *et al.*, 1992).

Extra-hemolymph pools of JH have been noted in the fat body, accessory sex glands, ovary and other undefined tissues, in *D. punctata* (Tobe *et al.*, 1984 and 1985) and *M. sexta* (Hammock *et al.*, 1975; Nowock *et al.*, 1976). The possible functions of

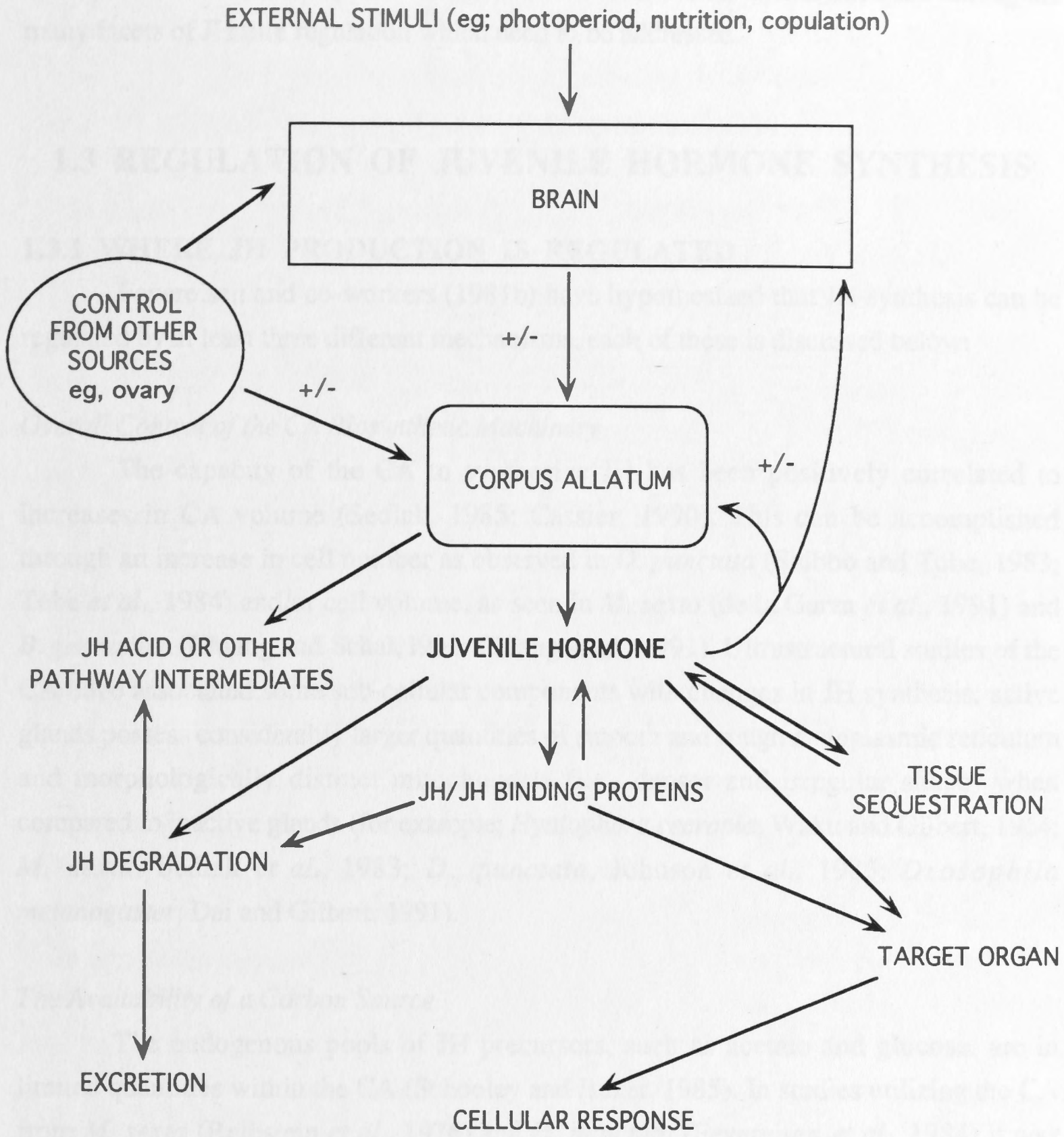


Figure 1.4; Factors which regulate *in vivo* JH titre in insects (adapted from; Williams, 1976; Cassier, 1990; Roe and Venkatesh, 1990).

these pools, their relative quantities and release of JH into the hemolymph are among the many facets of JH titre regulation which need to be addressed.

1.3 REGULATION OF JUVENILE HORMONE SYNTHESIS

1.3.1 WHERE JH PRODUCTION IS REGULATED

Feyereisen and co-workers (1981b) have hypothesized that JH synthesis can be regulated by at least three different mechanisms, each of these is discussed below:

Overall Control of the CA Biosynthetic Machinery

The capacity of the CA to synthesize JH has been positively correlated to increases in CA volume (Sedlak, 1985; Cassier, 1990). This can be accomplished through an increase in cell number as observed in *D. punctata* (Szibbo and Tobe, 1983; Tobe *et al.*, 1984) and/or cell volume, as seen in *M. sexta* (de la Garza *et al.*, 1991) and *B. germanica* (Chiang and Schal, 1991; Chiang *et al.*, 1991). Ultrastructural studies of the CA have associated some sub-cellular components with changes in JH synthesis; active glands possess considerably larger quantities of smooth and rough endoplasmic reticulum and morphologically distinct mitochondria (i.e., denser and irregular shape) when compared to inactive glands (for example; *Hyalophora cecropia*, Waku and Gilbert, 1964; *M. sexta*, Sedlak *et al.*, 1983; *D. punctata*, Johnson *et al.*, 1985; *Drosophila melanogaster*, Dai and Gilbert, 1991).

The Availability of a Carbon Source

The endogenous pools of JH precursors, such as acetate and glucose, are in limited quantities within the CA (Schooley and Baker, 1985). In studies utilizing the CA from *M. sexta* (Reibstein *et al.*, 1976) and *D. punctata* (Feyereisen *et al.*, 1984) it was observed that JH synthesis *in vitro* could only be maintained for a short period of time in the absence of JH precursors in the incubation medium.

Direct Control of a Small Number of Enzymes

Precise regulation of CA activity is most probably acting through the control of a small number of rate-limiting enzymes in the biosynthetic pathway and will determine the spontaneous activity of the gland (Feyereisen *et al.*, 1981a). Several groups have found that rate limitation may occur before the formation of mevalonate/homomevalonate (Pratt and Tobe, 1974; Baker *et al.*, 1983; Feyereisen and Farnsworth, 1987; Couillaud, 1991). The two enzymes which have been implicated as rate-limiting are HMG CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase (or HEG CoA (3-hydroxy-3-ethylglutaryl

coenzyme A) reductase in the case of the synthesis of the higher homologs of JH) and HMG/HEG CoA synthase (Figure 1.3). Other enzymes may also be rate-limiting in some instances (Cusson *et al.*, 1991), an obvious example being the absence of methyl transferase at particular stages in lepidopterans (see section 1.2.2).

HMG CoA reductase is rate-limiting in the vertebrate cholesterol biosynthetic pathway and is controlled by phosphorylation/dephosphorylation (Gibson and Parker, 1987; Goldstein and Brown, 1990). Reversible phosphorylation of HMG CoA/HEG CoA reductase has been observed in *M. sexta* (Monger and Law, 1982) with the activity of this enzyme being linked with fluctuations in JH synthesis in 5th instar larvae (Bhaskaran *et al.*, 1987). The competitive inhibitors of HMG CoA reductase, mevinolin and hydroxymethylglutarate, significantly decreased basal levels of JH synthesis in adult female *L. migratoria*, however upon the addition of mevalonate JH synthesis was restored, suggesting that mevalonate synthesis was rate-limiting (Applebaum *et al.*, 1991). Strong correlations between HMG CoA reductase activity and JH release were seen in CA from adult female *D. punctata* (Feyereisen and Farnsworth, 1987). However, there was no evidence for phosphorylation of the enzyme and the authors argued that HMG CoA reductase should not be considered rate-limiting because 50% of its enzymic activity could be lost without affecting JH synthesis (Feyereisen and Farnsworth, 1987).

An enzyme which may be tightly regulated in adult female *D. punctata* is HMG CoA synthase, as its activity decreases considerably faster than HMG CoA reductase when JH release is declining (Couillaud and Feyereisen, 1991). This is not the case in adult female *L. migratoria* where removal of JH stimulatory factors via severance of the NCA-I results in cessation of JH release from the CA within two days but no detectable decrease in HMG CoA synthase activity over the same period (Couillaud and Rossignol, 1991).

Clearly the rate-limiting enzymes in the JH biosynthetic pathway may vary between species and many more studies on each enzyme in the pathway in other species are required before any generalization on the enzymatic targets of factors regulating the JH biosynthetic pathway can be made.

1.3.2 HOW JH PRODUCTION IS REGULATED

Since the discovery of neurosecretory axons innervating the retrocerebral complex in insects (Stumm-Zollinger, 1957) neurohemal secretions have been implicated in the regulation of JH release. Neuropeptides may stimulate (termed allatotropins), inhibit (allatinhibins) or maintain low rates of (allatostatins) JH synthesis. These peptides can be delivered directly to the CA via neurosecretory cell (NSC) axons or reach the CA via the hemolymph after release from an endocrine organ such as the CC (Tobe and Stay, 1985). It is possible that conventional neuron firing is also involved in the regulation of

JH synthesis, perhaps controlling the release of neurosecretory material (Tobe and Stay, 1985).

Hormones produced in tissues other than the brain, including the ovary and the prothoracic gland, may also influence JH production (Tobe and Stay, 1985). In many cases it is unclear whether regulation occurs via direct action on the CA or indirectly through the brain. In fact JH itself has been implicated in the regulation of its own synthesis through both negative and positive feedback mechanisms (Tobe and Stay, 1985). For example JH release can be reduced by 45% from the CA of *Blattella germanica* in the presence of 300μM JH III (Maestro *et al.*, 1993).

The relative roles of the abovementioned factors in modulating JH synthesis vary among insect species and even between developmental stages within a species. Factors such as nutrition, matedness and photoperiod may also play a role in the pattern of JH control (Tobe *et al.*, 1977; Tobe, 1980; Feyereisen, 1985b; Tobe and Stay, 1985; Khan, 1988; Goodman, 1990; Raabe, 1989). Generalizations are difficult to make and so a few specific examples are given below for the three species which have been studied most intensively.

1.3.2.1 REGULATION OF ADULT FEMALE *D. punctata* CA

The presence of a spermatophore in the bursa is believed to relieve inhibition of the CA mediated by the brain in female *D. punctata* (Stay and Tobe, 1977 and 1978). The resulting increase in JH production is not only due to the removal of inhibition by the brain, but also the presence of a stimulatory hormonal effect from the ovary (Rankin and Stay, 1983; Tobe *et al.*, 1984) and possibly the brain (Stay and Tobe, 1977).

The low levels of JH production observed in virgin females and during pregnancy is controlled by a family of allatostatins, of which five have now been characterized at the amino acid level (Figure 1.5). These peptides have the same three carboxyl terminal amino acids, share a common tyrosine residue, are amidated (Pratt *et al.*, 1989 and 1991; Woodhead *et al.*, 1989), and act in a dose dependent, reversible manner (Woodhead *et al.*, 1989; Pratt *et al.*, 1991).

Each allatostatic peptide is able to inhibit JH production to some degree from isolated CA of adult female *D. punctata* (Woodhead *et al.*, 1989; Pratt *et al.*, 1989). The sensitivity of the CA to these peptides varies considerably during adult female life, suggesting regulation of allatostatin receptors or subsequent intracellular events during the *D. punctata* reproductive cycle (Stay *et al.*, 1991). Allatostatins Dip-ASA1-4 (Figure 1.5) also inhibit JH synthesis from *D. punctata* larval CA (Stay *et al.*, 1991) and Dip-ASA1 reduces JH production from adult female CA of another cockroach, *P. americana* (Weaver, 1991). Other *D. punctata* allatostatins have not been tested in such studies. Interestingly, related peptides have been isolated from the dipteran, *Calliphora vomitoria,*

***D. punctata* Allatostatins;**

H-Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-NH2 (Dip-ASA1)

H-Gly-Asp-Gly-Arg-Leu-Tyr-Ala-Phe-Gly-Leu-NH2 (Dip-ASA2)

H-Gly-Gly-Ser-Leu-Tyr-Ser-Phe-Gly-Leu-NH2 (Dip-ASA3)

H-Asp-Arg-Leu-Tyr-Ser-Phe-Gly-Leu-NH2 (Dip-ASA4)

H-Ala-Tyr-Ser-Tyr-Val-Ser-Glu-Tyr-Lys-Arg-Leu-Pro-Val-Tyr-Asn-Phe-Gly-Leu-NH2 (Dip-ASB2)

***M. sexta* Allatotropin;**

H-Gly-Phe-Lys-Asn-Val-Glu-Met-Met-Thr-Ala-Arg-Gly-Phe-NH2 (Mas-AT)

***M. sexta* Allatostatin;**

pGlu-Val-Arg-Phe-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile-Ser-Cys-Phe-OH (Mas-AS)

Figure 1.5; Peptide sequences of all neuropeptides that influence juvenile hormone synthesis that have been characterized at the amino acid level. Mas-AT was isolated by Kataoka *et al.* (1989), Mas-AS by Kramer *et al.* (1991), Dip-ASA1-4 by Woodhead *et al.* (1989) and Dip-ASB2 by Pratt *et al.* (1991).

which have allatostatic activity on CA from adult female *D. punctata* but no effect on JH synthesis by the CA of larval or adult *C. vomitoria* (Duve *et al.*, 1993).

Recent immunohistochemical data suggests the allatostatic peptide family has numerous roles in the insect, with extensive immunoreactivity to Dip-ASA1 having been detected in the brain of adult female *D. punctata,* including NSC axons from the pars lateralis arborizing in the CC and CA (Stay *et al.*, 1992).

1.3.2.2 REGULATION OF ADULT FEMALE *L. migratoria* CA

The glandular activity of the CA in adult female *L. migratoria* is regulated by at least four different factors. During normal adult female development, JH production is stimulated and maintained in the long term by direct input from the brain to the CA via the nervi corpori cardiaci II (NCC II) and nervi corpori allati I (NCA I) (Ferenz and Kaufner, 1981; Couillaud and Girardie, 1986-1987), and in the short term by an allatotropin released into the hemolymph at the CC (Gadot *et al.*, 1987a; Couillaud and Girardie, 1990). An ovarian derived allatostatin is believed to be responsible for a decrease in JH synthesis following vitellogenesis (Applebaum *et al.*, 1991). Neural inhibition of adult female CA has been observed during imaginal diapause and appears to be controlled through lateral neurosecretory cells of the protocerebrum (Poras *et al.*, 1983).

Extracts of the CC and brain from adult female and male *L. migratoria* stimulate the synthesis of JH by the CA of adult females of various ages (Ferenz and Diehl, 1983; Gadot and Applebaum, 1985; Lehmberg *et al.*, 1992). This stimulation is dose dependent (Ferenz, 1984; Gadot *et al.*, 1987a) and rapidly reversible (Gadot and Applebaum, 1985). Partial purification of the allatotropin reveals it to have a molecular weight of 700-1500 (Rembold *et al.*, 1986). Similar levels of stimulation have been noted with FA and extracts prepared from *L. migratoria* brains, suggesting the allatotropic factor may control rate-limiting enzyme(s) early in the JH biosynthetic pathway (Gadot and Applebaum, 1986). The inability of the allatotropin to rescue JH synthesis in the presence of competitive inhibitors of HMG CoA reductase, whereas the biosynthetic intermediate mevalonate could, indicates that the allatotropin may activate JH production through the phosphorylation of HMG CoA reductase (Applebaum *et al.*, 1991).

1.3.2.3 REGULATION OF FIFTH INSTAR LARVAL *M. sexta* CA

Regulation of JH production by CA of fifth instar larval *M. sexta* is a very complex process (Granger and Janzen, 1987; Bollenbacher, 1988; Janzen *et al.*, 1991) and many factors controlling JH release are yet to be identified. For a comprehensive review of JH and ecdysteroid fluctuations, and their suspected roles, during the fifth larval instar of *M. sexta* refer to Bollenbacher (1988).

At day 0 of the fifth larval instar of *M. sexta*, JH production from the CA is relatively high and is believed to be the result of stimulation by an allatotropin(s) (Granger and Janzen, 1987). During days 1-2 of the fifth instar, JH release decreases, which is at least partly due to a JH I specific allatostatin (Granger and Janzen, 1986). Between days 2-5, JH synthesis decreases further and gradually changes to JH acid synthesis, as a result of the non-reversible action of an allatinhibin which is most likely regulating JH acid methyl transferase activity (Bhaskaran *et al.*, 1990). A second, post pupal commitment JH peak (predominantly JH acid) during days 6-7 (Bollenbacher, 1988) is indirectly initiated by 20-hydroxyecdysone (Whisenton *et al.*, 1987) which acts on the brain to stimulate JH/JH acid synthesis via nerves innervating the CA (Granger *et al.*, 1984). On days 8-9 JH release decreases, with pupal ecdysis occurring around days 9-10 (Bollenbacher, 1988). A JH III specific allatotropin which is probably produced in the lateral NSC of the protocerebrum has been reported in day 0 pupae, but the physiological role of this observation is unknown (Bollenbacher *et al.*, 1984).

1.3.2.4 REGULATION OF ADULT FEMALE *M. sexta* CA

JH synthesis in adult female *M. sexta* is initiated around eclosion and requires intact NCC I/II (Nijhout and Riddiford, 1974; Sasaki and Riddiford, 1984; Ishizaki *et al.*, 1987). However it has not been determined whether this activation requries the NCA I to be intact.

The first evidence presented for an allatotropin in adult female *M. sexta* was the publication of the peptide sequence of this factor (Kataoka *et al.*, 1989). This 13 amino acid peptide (Figure 1.5) was purified from pharate adult heads and elicits a dose dependent increase in JH synthesis by CA of 0-4 hour old female *M. sexta*. The C-terminal octapeptide-amide appears to be essential for biological activity (Kataoka *et al.*, 1989).

The *M. sexta* allatotropic factor (Mas-AT) is unable to stimulate JH synthesis either by CA of larval or pupal *M. sexta,* or by CA of adult female *T. molitor, Schistocerca nitens* or *P. americana*. However, Mas-AT does significantly stimulate JH production from CA of adult female *He. virescens,* leading the authors to conclude that the activity of this peptide may be restricted to adult female Lepidoptera (Kataoka *et al.*, 1989).

The Mas-AT stimulates the synthesis of JH I, II and III in adult female *M. sexta*, with a slightly greater stimulation of JH II (Unni *et al.*, 1991). The effect of the peptide is rapidly reversible *in vitro* (Unni *et al.*, 1991) and may act via the inositol 1,4,5-trisphosphate pathway (Reagan *et al.*, 1992).

In addition to the allatotropin, a CA inhibitory peptide has also been isolated from pharate adult *M. sexta*. This allatostatin (Mas-AS) has been characterized as a 15 amino

acid peptide (see Figure 1.6) that inhibits JH synthesis by CA of fifth instar larvae and adult female *M. sexta* in a dose dependent manner. JH production was also inhibited from adult female *He. virescens* CA, but not adult female CA from *T. molitor, P. americana* or *Melanoplus sanguinipes*. Therefore, as with Mas-AT, the biological activity of Mas-AS maybe restricted to lepidopterans (Kramer *et al.*, 1991). The free acid and amidated forms of Mas-AS have equal potency (Kramer *et al.*, 1991), but the homolog specificity of Mas-AS if any, has not been reported. Addition of FA to *in vitro* assays containing the inhibitory factor has a "rescuing" effect, implying that the allatostatin works through rate-limiting steps prior to the two terminal enzymatic steps. Unlike the situation in adult female *D. punctata* (see section 1.3.2.1) there appears to be only one allatostatin in pharate adult *M. sexta* heads.

The presence of CA inhibitory and stimulatory peptides in the brain of pharate adult *M. sexta* raises the question of their respective functions at this developmental stage. Mas-AS may ensure low quantities of JH during pupal development, with pharate adulthood being a transition period where Mas-AS production is being switched off and Mas-AT production switched on. It is possible that the relative quantities of Mas-AS and Mas-AT determine JH release from the CA, where Mas-AS production is maintained during prolonged virginity or low food levels. The homolog specificity of Mas-AS is yet to be reported, however if Mas-AS does inhibit the synthesis of specific JH homologs it may act in the regulation of JH homolog ratios in adult *M. sexta*.

1.4 NEUROPEPTIDE PROCESSING

Neuropeptides are usually contained within large precursor proteins. A series of post-translational processing events is required for the production of mature, biologically active peptides (Douglass *et al.*, 1984; Loh *et al.*, 1984; Lynch and Snyder, 1986; Thomas *et al.*, 1988; Sossin *et al.*, 1989). The active peptide may only constitute a fraction of the preprohormone sequence; for example active bradykinin represents only 2% of the preprohormone sequence in bovines (Nawa *et al.*, 1983).

The number of peptides encoded by a single gene varies considerably from one, as seen in the *M. sexta* eclosion hormone gene (Horodyski *et al.*, 1989), up to 28 in the case of the *Aplysia* FMRFamide preprohormone gene (Schaefer *et al.*, 1985). A single gene encoding multiple peptides may consist of two or more copies of the same peptide and/or different physiologically active peptides (Amara *et al.*, 1982; Douglass *et al.*, 1984; Taussig and Scheller, 1986; Sossin *et al.*, 1989), as observed in the *Aplysia* FMRFamide gene (Schaefer *et al.*, 1985). Related peptides can be also be encoded on different preprohormone genes as seen in the opioid peptide family (see Douglass *et al.*,

1984).

A diagram illustrating the main structural features of neuropeptide preprohormones and their processing signals is presented in Figure 1.6; details of these features are given below.

1.4.1 SIGNAL SEQUENCE

The first process in preprohormone processing is the co-translational removal of the N-terminal signal sequence. This 20-30 amino acid region contains a central hydrophobic core which directs the preprohormone into the endoplasmic reticulum (Docherty and Steiner, 1982; Douglass *et al.*, 1984; Sossin *et al.*, 1989). The sequence is usually removed and rapidly degraded before the completion of translation (Docherty and Steiner, 1982; Douglass *et al.*, 1984). Structurally distinct regions within the hydrophobic signal sequence direct signal peptide cleavage (von Heijne, 1983). von Heijne (1986) has devised an algorithm based on known preprohormone cleavage sites which predicts the cleavage site with a 75-80% accuracy, although the exact sites of signal peptide cleavage need to be determined for individual preprohormones via the isolation of processing intermediates.

1.4.2 PROTEOLYTIC CLEAVAGE

Peptides are liberated from the prohormone via proteolytic cleavage. Sites of cleavage are commonly dibasic pairs of amino acids flanking the peptide sequence, usually lysine-lysine or lysine-arginine; however mono-basic (usually arginine), tribasic and tetrabasic cleavage sites have also been found. Cleavage appears to be a two step process (Figure 1.6) with trypsin-like endopeptidases cleaving on the C-terminal side of the basic residues and then carboxypeptidase enzymes removing the basic amino acid(s) which remain attached to the peptide sequence. Proteolytic cleavage occurs in the Golgi apparatus and/or the secretory vesicles. Evidence suggests that the endopeptidases have a broad cleavage specificity and are able to process a wide variety of prohormones (see Schwartz, 1986; Fricker, 1988; Thomas *et al.*, 1988; Sossin *et al.*, 1989; Masler *et al.*, 1993).

Only about 10% of the single basic amino acids within a prohormone will act as cleavage sites, compared to almost every dibasic amino acid sequence (Devi, 1991). The surrounding sequence and tertiary structure are believed to be important in governing which single basic amino acids are used as endopeptidase cleavage sites (Devi, 1991; Nakayama *et al.*, 1992). Examination of known mono-arginyl cleavage sites (Devi, 1991; Nakayama *et al.*, 1992), combined with *in vitro* studies of the endopeptidase cleavage of prorenin mutants in mouse pituitary AtT-20 cells (Nakayama *et al.*, 1992) has resulted in the identification of some of the sequence constraints (or what the authors call 'rules') for

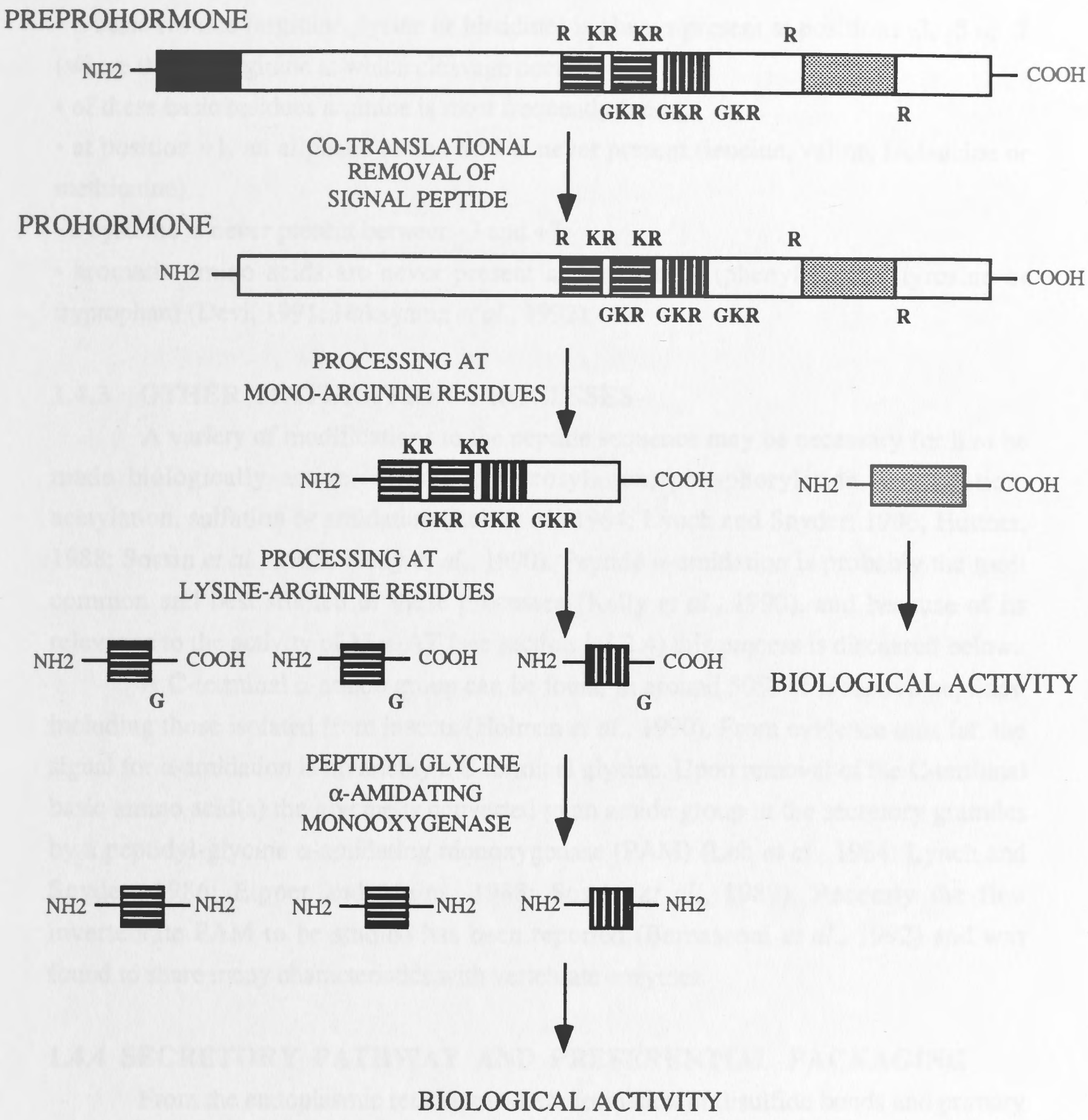


Figure 1.6; Some aspects and characteristics of neuropeptide preprohormone processing (adapted from Sossin *et al.*, 1989). Those amino acids shown above the preprohormone/prohormone represent residues immediately N-terminal to the active neuropeptides, whereas amino acids below the preprohormone/prohormone structures represent residues immediately C-terminal to the active peptides. The carboxypeptidase removal of basic residues from the C-terminal end of the peptide after endopeptidase cleavage as described in the text (section 1.4.2) is not shown. The black box at the N-terminal end of the preprohormone represents the hydrophobic signal sequence. All other boxes represent neuropeptides.

mono-arginyl cleavage. These are;

• a basic residue (arginine, lysine or histidine) is always present at positions -3, -5 or -7 (where 0 is the arginine at which cleavage occurs),

• of these basic residues arginine is most frequently found,

• at position +1, an aliphatic amino acid is never present (leucine, valine, isoleucine or methionine),

• a cysteine is never present between -3 and +7,

• aromatic amino acids are never present at position -1 (phenylalanine, tyrosine or tryptophan) (Devi, 1991; Nakayama *et al.*, 1992).

1.4.3 OTHER MATURATION PROCESSES

A variety of modifications to the peptide sequence may be necessary for it to be made biologically active, including glycosylation, phosphorylation, methylation, acetylation, sulfation or amidation (Loh *et al.*, 1984; Lynch and Snyder, 1986; Huttner, 1988; Sossin *et al.*, 1989; Kelly *et al.*, 1990). Peptide α-amidation is probably the most common and best studied of these processes (Kelly *et al.*, 1990), and because of its relevance to the activity of Mas-AT (see section 1.3.2.4) this process is discussed below.

A C-terminal α-amide group can be found in around 50% of bioactive peptides, including those isolated from insects (Holman *et al.*, 1990). From evidence thus far, the signal for α-amidation is invariably a C-terminal glycine. Upon removal of the C-terminal basic amino acid(s) the glycine is converted to an amide group in the secretory granules by a peptidyl-glycine α-amidating monoxygenase (PAM) (Loh *et al.*, 1984; Lynch and Snyder, 1986; Eipper and Mains, 1988; Sossin *et al.*, 1989). Recently the first invertebrate PAM to be studied has been reported (Bernasconi *et al.*, 1992) and was found to share many characteristics with vertebrate enzymes.

1.4.4 SECRETORY PATHWAY AND PREFERENTIAL PACKAGING

From the endoplasmic reticulum where formation of disulfide bonds and primary N-linked glycosylation may occur, the prohormone is transported to the *cis* aspect of the Golgi apparatus in small vesicles where further processing takes place. In the Golgi apparatus peptides are targetted into secretory vesicles which are transported to the NSC terminals for release (Sossin *et al.*, 1989).

With many examples of different peptides being encoded on the same preprohormone gene and also different preprohormones being expressed in the same NSC (Hökfelt *et al.*, 1978; Chan-Palay, 1987) one of the most interesting questions about neuropeptide processing is whether specific peptides can be packaged and released independently. Chung *et al.* (1989) have identified prohormone binding proteins, termed sortases, in the Golgi. These proteins could produce preferential packaging of peptides.

Bioactive peptides produced from the egg laying preprohormone in the bag cell neurons of *Aplysia californica* have been found to be differentially packaged (Fisher *et al.*, 1988; Newcomb *et al.*, 1988; Sossin *et al.*, 1990b) and spatially separated to autocrine and hormonal release sites within the same NSC (Sossin *et al.*, 1990a).

1.5 *HELICOVERPA ARMIGERA*

1.5.1 DISTRIBUTION AND TAXONOMY

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a moth which has a wide geographic distribution, being endemic to Australia, New Zealand, Asia, Africa and southern Europe (Hardwick, 1965; Reed and Pawar, 1982). As a consequence of its widespread occurrence *H. armigera* has many common names (Broadley, 1977), being referred to most frequently as the cotton bollworm.

Since Hardwick (1965) divided the existing *Heliothis* genus into two parts, introducing the new generic name *Helicoverpa,* there has been considerable confusion over the naming of *H. armigera* (Nye, 1982; Gupta, 1986; Mitter *et al.*, 1993). Only 2% of the 1386 scientific papers published on the species between 1970 and 1984 have used the new genus name *Helicoverpa* (Gupta, 1986). Nevertheless, in this thesis I shall follow the classification of Hardwick (1965) and Matthews (1991) and the species will be referred to as *Helicoverpa armigera.*

Within the lepidopteran family Noctuidae, *Helicoverpa* is situated in the Heliothinae sub-family which contains approximately 400 species (Matthews, 1991). Three other species in the family are major economic pests and consequently have received the greatest amount of attention. These are *Helicoverpa punctigera,* which is found solely in Australia (Hardwick, 1965), and *Helicoverpa zea* and *Heliothis virescens* both of which are endemic to North and South America (Hardwick, 1965; Todd, 1978).

1.5.2 LIFE CYCLE

H. armigera is a holometabolous insect, with development consisting of five larval instars, a pupal stage and finishing life as an adult moth (Hardwick, 1965, Zalucki *et al.*, 1986). In a study conducted in Queensland, Kirkpatrick (1962) observed *H. armigera* to emerge in September/October after overwintering diapause and complete 5-7 generations before the induction of diapause around April of the following year.

As for most insect species, the length of different developmental stages in *H. armigera* is highly correlated to factors such as temperature (Twine, 1977; Foley, 1981; Room, 1983; Ellington and El-Sokkari, 1986), nutrition (Lukefahr and Martin, 1964; Kay *et al.*, 1979) and relative humidity (Ellington and El-Sokkari, 1986).

Kirkpatrick's (1962) study of the life history of *H. armigera* in the field uncovered large individual variations of developmental rates within and between different times of the year. Under optimized constant laboratory conditions (see section 2.2.1) eggs hatch 3-4 days after oviposition, larval development takes around 22 days, the pupal phase lasts 13 days and the adult moth will survive for approximately 14 days (personal observation).

Adult *H. armigera* are extremely fecund (Lingren *et al.*, 1982; Zalucki *et al.*, 1986; Matthews, 1991), each female producing an average of 1702 eggs (Hardwick, 1965), oviposited one at a time (Fitt, 1989). Oviposition occurs predominantly at night and is reduced by moonlight (Persson, 1974).

1.5.3 PEST STATUS

H. armigera, H. punctigera, H. zea and *He. virescens* are considered major economic pests (Zalucki *et al.*, 1986; Fitt, 1989), with *H. armigera* having one of the broadest geographic distributions of any agricultural pest (Fitt, 1989). Economic losses are enormous and result from decreased crop yields and increased costs involved in pest control. Exact and current figures for their overall economic damage are hard to come by (Pimental *et al.*, 1992), but a few examples include; in excess of $1 billion for control and damage in the United States as result of *H. zea* and *He. virescens* (USDA, 1976), in excess of $300 million damage to chickpea and pigeonpea crops alone due to *H. armigera* in India (Reed and Pawar, 1982), and an estimated cost of control of $23.5 million in numerous crop species in Australia for *H. armigera* and *H. punctigera* (Wilson, 1982).

A number of factors contribute to the destructive characteristics of these moth species;

a) They are highly polyphagous (Wilson, 1982; Zalucki *et al.*, 1986; Fitt, 1989). Apart from many non-agriculturally important plant species (Zalucki *et al.*, 1986), *H. armigera* consumes an impressive array of food, fibre, fodder, horticultural and ornamental crops (Fitt, 1989), both monocotyledons and dicotyledons (Zalucki *et al.*, 1986).

b) The adults are extremely mobile (Hendricks *et al.*, 1973; Haile *et al.*, 1975; Farrow and Daly, 1987) ensuring rapid spread of the species into areas where numbers may have declined.

c) Their high fecundity (see section 1.5.2).

d) Their ability to undergo both winter and summer diapause when conditions are unfavourable (Wilson *et al.*, 1979; Fitt, 1989).

e) The impact of the above characters is enhanced by the widespread levels of resistance of *H. armigera* (Goodyer *et al.*, 1975; Gunning *et al.*, 1984; Sawicki and Denholm, 1987; Daly and Fisk, 1992), *H. zea* (Sparks, 1981) and *He. virescens*

(Sparks, 1981; Luttrell *et al.*, 1987) to a large range of chemical pesticides. In fact, the pest status of *He. virescens* has been attributed to pesticides causing the destruction of natural enemy populations (Reed and Pawar, 1982).

1.6 EXPERIMENTAL INTRODUCTION

In the five following chapters I will present and discuss my findings on the neuroendocrine regulation of JH production in adult female *Helicoverpa armigera*. Chapter 2 addresses the role of JH in *H. armigera* female reproduction, describes the establishment of an *in vitro* radiochemical assay (RCA) to measure JH production, and examines some aspects of JH physiology in adult female life. The third chapter deals with the control of JH synthesis, where the RCA described in chapter 2 was used to establish that neuroendocrine factors stimulate JH release from the CA. Chapter 4 describes the utilization of molecular biology techniques to isolate and partially characterize the genes encoding the *H. armigera* allatotropic factor (Hea-AT) and the *M. sexta* allatotropic factor (Mas-AT). Experiments studying the spatial expression of the Hea-AT gene are presented in chapter 5, with the sixth and final chapter being a general discussion of the work.

CHAPTER 2

EGG MATURATION AND JUVENILE HORMONE RELEASE IN VIRGIN FEMALE *Helicoverpa armigera*

CHAPTER 2

2.1 INTRODUCTION

Lepidopteran species can be divided into two broad groups on the basis of their endocrine requirements for female reproduction (section 1.1.3.2.3), with one needing ecdysteroids for egg maturation (group I), the other JH (group II). Thus, before details of the neuroendocrine regulation of egg maturation in *Helicoverpa armigera* were addressed, the broad strategy of endocrine control was investigated. *H. zea* and *He. virescens*, two close relatives of *H. armigera,* both have a strong dependence on JH for oocyte development (Ramaswamy and Cohen, 1991; Satyanarayana *et al.*, 1991) and it is highly likely that *H. armigera* possesses a similar endocrine control of egg production.

JH production by the CA of insects has been most widely studied using an *in vitro* radiochemical assay (RCA) (Baker, 1990). This assay was established by Judy *et al.* (1973a,b) and relies on the incorporation of a radioactively labelled (usually ^{3}H) methyl group from methionine into JH, late in the biosynthetic pathway (see Figure 1.3 and Appendix 3) (Feyereisen, 1985a; Tobe and Stay, 1985; Baker, 1990). Radioisotope incorporation occurs at a 1:1 (label:JH) molar ratio in all species examined (Judy *et al.*, 1973a,b; Tobe and Pratt, 1974; Pratt and Bownes, 1977; Weaver *et al.*, 1980; Feyereisen *et al.*, 1981a; Cusson *et al.*, 1990) indicating that methionine is effectively the sole source of the methyl group incorporated into JH acid to produce JH.

Some aspects of the regulation of JH synthesis can be studied by experiments utilizing exogenous JH precursors, such as farnesoic acid (FA) (Tobe and Pratt, 1976; Feyereisen, 1985b). In most insects thus far examined, the last two steps of JH production (see Figure 1.3) are not believed to be rate-limiting (Feyereisen, 1985b; Yagi *et al.*, 1991) and so the rate of conversion of FA to JH III can be taken as the maximum potential of the CA to synthesize JH (Gadot *et al.*, 1989). The extent to which FA stimulates JH III synthesis has been expressed as the Fractional Endocrine Activity Ratio (FEAR), calculated by dividing the basal rate of JH release by the rate of JH release in the presence of FA (Tobe and Pratt, 1976). A FEAR value near 1 implies that the flux through the rate-limiting step(s) in the JH biosynthetic pathway is close to the biosynthetic capacity of the two terminal steps (Feyereisen, 1985b).

2.2 MATERIALS AND METHODS

2.2.1 Insect Rearing

H. armigera were reared at a constant temperature of 25°C and exposed to a 14 hour light, 10 hour dark photoperiod (lights off 2030, lights on 0630). Larvae were reared on a soybean/wheatgerm based diet (Teakle and Jensen, 1985). Early developmental stages (1st-3rd instar larvae) were kept in paper cups (45 x 75 mm) at a density of approximately 50 per cup. Individual third instar larvae were transferred to containers (27 x 45 mm) to complete their development in isolation to prevent cannibalism. After pupation the animals were washed in bleach (5%v/v) to remove external microorganisms and held in plastic containers until adult emergence.

Adults were maintained in mesh cages (20 x 25 cm), fed a honey solution (Teakle and Jensen, 1985) and eggs were collected approximately every second day to produce the next generation of moths.

For experimental animals, newly eclosed virgin females were collected at 0830 hours (previous 12 hours; 10 dark and two light) and 2030 hours (previous 12 hours all light). Moths emerging in any given 12 hour period were kept as a group and aged for various periods before use. Therefore, in the figures presented in the Results section (2.3), values on the x-axis (time) represent the median age of the particular cohort of moths used for that data point. For example, adult female moths 0-12 hours of age appear at the median age of 6 hours.

Two laboratory strains of *H. armigera* were used in this study, SUS (susceptible to pyrethroids) and AN (resistant to pyrethroids). These strains have been described previously by Daly and Fisk (1992) where they refer to the AN strain as 'field'. All experiments, except where indicated, were carried out using moths of the AN strain.

2.2.2 Dissections

H. armigera were anaesthetized with CO_2, submerged in *Manduca* saline (1.6mM Hepes (pH 6.8), 25mM NaCl, 25mM KCl, 15mM $MgCl_2$, $CaCl_2$ and 150mM sucrose; Nijhout and Riddiford, 1979) and the retrocerebral complex removed with the aid of a dissecting microscope. No attempt was made to separate the CC from the CA, or to completely remove any surrounding tissue, as this has been reported to damage CA cells, adversely affecting JH synthesis (Tobe and Stay, 1985; Cusson *et al.*, 1990). In experiments using tissues other than the retrocerebral complex, a volume of material approximately equivalent to the CC-CA was removed.

The basal oocytes, defined as the most posterior oocytes in the calyx (Callahan, 1958), were removed and their diameter measured with a micrometer attached to the eye piece of a dissecting microscope.

2.2.3 Decapitation Experiment

One hour after eclosion, virgin female *H. armigera* were decapitated and the wound sealed with vacuum grease. The animals were injected with 5µl of safflower oil containing JH III (a gift of Dr R. Rickards, Research School of Chemistry, Australian National University) or the JH mimic, methoprene (a gift of Dr G. Staal, Zoecon Corp., Palo Alto, USA), at the concentrations specified in Table 2.1. Control decapitated moths were injected with 5µl of safflower oil only. Undecapitated control animals were not fed. At two days of age the ovaries were dissected out and the number of mature eggs counted under a dissecting microscope.

2.2.4 Radiochemical Assay

The RCA medium consisted of medium TC199 with Earle's salts, 1.46mM L-glutamine and 20mM Hepes, without methionine (Cytosystems), supplemented with 61µM sodium propionate, 72µM calcium chloride, 250µM L-methionine, 200µM L-glutamine, with the pH adjusted to 6.5 with 2M HCl. After sterilization through a 0.45µM nitrocellulose filter (Sartorius), Ficoll 400 was added to a final concentration of 2% (w/v). Each assay contained 7.7×10^4Bq of L-[methyl-^{3}H] methionine (specific activity 3.1×10^{12}Bq/mmole, Amersham) at a final specific activity of 6.2×10^9Bq/mmole.

CA were placed singly in small Carbowax treated glass tubes (50mm x 6mm) in 50µl of RCA medium and incubated for four hours in the dark at 28°C with gentle agitation. The glands were then removed and the media extracted with 250µl of iso-octane as described by Feyereisen and Tobe (1981). In this system, JH partitions into the organic phase whereas the unincorporated labelled methionine remains in the aqueous phase. A total of 150µl of the organic (upper) phase was added to 3ml of scintillation fluid (Optiphase 'Hisafe' II-LKB) and the radioactivity quantified by liquid scintillation spectrometry (Model LS 2800-Beckman).

In the experiment to measure linearity of JH release with time *in vitro*, the medium was removed and extracted each hour, and the CA were placed in fresh medium to continue the incubation.

To measure the Fractional Endocrine Activity Ratio (FEAR), farnesoic acid was added to the RCA medium to produce a final concentration of 20µM. After a 1 hour pre-incubation, the basal level of JH release was determined over a 1.5 hour period. Stimulation of JH release was measured by a further 1.5 hour incubation after the addition of FA.

2.2.5 Quench Curve Construction

A quench curve for JH was constructed using NaOH as a quenching agent and

known quantities of chain labelled JH III (see Appendix 3) (New England Nuclear) as described by Peng (1977).

The data collected is described by a simple linear regression model. The regression equation is:

$$Y = 0.42443 - (1.6078 \times 10^{-3})X. \qquad (R^2 = 0.951)$$

where Y is the efficiency of counting and X is the H value from the scintillation counting output (H value is a measure of quench produced from an external radioactive Cs source counted next to or through the sample by the instrument). In most experimental cases H was between 29 and 31, indicating that the counts observed were approximately 37% of the counts present. All radioactivity measurements have been quench corrected before calculating the rate of JH release (pmoles JH/hr/CA).

2.2.6 Resolution of JH Homologs

For experiments in which JH and related metabolites were analysed by thin layer chromatography (TLC), the specific activity of the L-[methyl-^{3}H]-methionine was increased to 1.9×10^{10}Bq/mmole and two pairs of glands were incubated per tube. Extractions were done with hexane, rather than iso-octane, because its higher volatility allowed more rapid loading on the TLC plates.

Approximately 200µl of the extracted hexane was loaded directly onto F254 silica gel 60, 20cm x 20 cm, plastic backed plates (Merck). The TLC plate was developed with 30% ethyl acetate, 70% hexane and the solvent front was allowed to migrate until it was approximately 2cm from the top of the plate. The plate was air dried, divided into 1cm (wide) x 0.5cm (high) strips, the strips were added to scintillation fluid and the radioactivity quantified as described for the RCA (section 2.2.4).

The position of individual JH metabolites was determined by running cold standards consisting of JH I, II and III, and their respective diols (a gift of Dr R. Rickards, RSC, ANU) on either side of the test lanes. Migration of the cold standards was detected colourimetrically by immersion of the lanes containing the cold standards in 5% phosphomolybdic acid (in ethanol) followed by heating at 60°C for ten minutes.

2.3 RESULTS

2.3.1 Oocyte Maturation

The basal oocyte diameter in virgin female *H. armigera* increased rapidly over the first day of adult life (Figure 2.1), reaching maximum size by two days of age.

At two days of age, unfed virgin *H. armigera* possessed an average of 37 mature oocytes in their ovarioles (Table 2.1). Decapitation of newly eclosed moths, which

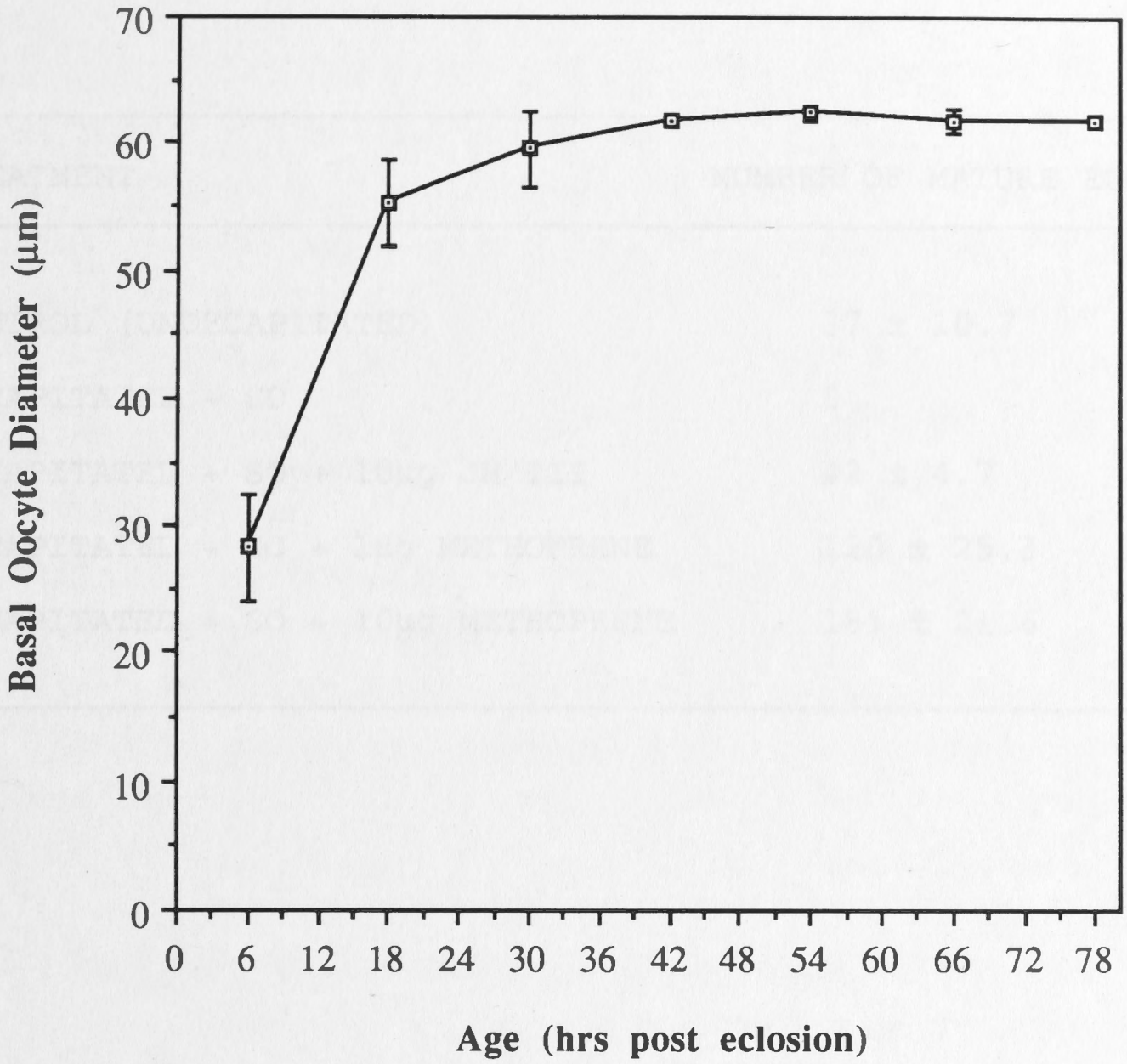


Figure 2.1; Increase in basal oocyte diameter of adult virgin female *H. armigera* plotted against time of eclosion. Values are expressed as means ± SEM of 6 replicates.

TREATMENT	NUMBER OF MATURE EGGS
CONTROL (UNDECAPITATED)	37 ± 10.7
DECAPITATED + SO	0
DECAPITATED + SO + 10µg JH III	42 ± 4.7
DECAPITATED + SO + 1µg METHOPRENE	120 ± 25.3
DECAPITATED + SO + 10µg METHOPRENE	181 ± 21.6

Table 2.1; Egg maturation in decapitated virgin *H. armigera* after the injection of JH III or the JH analog, methoprene. Values are expressed as means ± SEM of 6-12 replicates. SO = Safflower Oil.

removes both the brain and the retrocerebral complex, invariably resulted in failure to produce any mature eggs in the ovarioles after two days. The injection of 10μg of JH III dissolved in safflower oil restored egg maturation to a level approximately equal to the undecapitated control animals. The JH analog methoprene produced a significantly greater number of mature oocytes than the undecapitated control, with the injection of 10μg resulting in the production of more mature eggs than 1μg (Table 2.1).

2.3.2 Influence of L-Methionine Concentration on Rate of JH Release

The effect of varying the concentration of L-methionine in the RCA medium (section 2.2.4) on JH release from CA of virgin adult female *H. armigera* is presented in Figure 2.2. No significant difference in JH production was observed between concentrations of 100 and 250μM L-methionine. A concentration of 250μM L-methionine was used in the standard RCA medium, to reduce the likelihood of the substrate becoming limiting during the course of an assay.

2.3.3 Influence of Medium pH on Rate of JH Release

Media with pH values ranging from 5 to 7.5, in 0.5 increments, were tested in the standard assay (section 2.2.4). A pH value of 6.5 gave optimal rates of JH release from CA of virgin adult female *H. armigera* (Figure 2.3), although the rates did not vary significantly for pH values between 6 and 7. A pH of 6.5 was chosen for the optimized assay (section 2.2.4).

2.3.4 Time Course of ^{3}H-Labelled JH Release from the CA

A total of nine individual CA were examined for their rate of incorporation of the radio-labelled methyl group from methionine into JH over the duration of a typical assay. In both low activity glands (from moths under 12 hours old) (Figure 2.4A) and high activity glands (from moths 2-3 days of age) (Figure 2.4B) the release of labelled JH from the CA was essentially linear for at least five hours.

2.3.5 Effect of Sodium Propionate in the Media on JH Release

Cusson and coworkers (1990) studying adult females of the noctuid moth, *P. unipuncta* added 61μM sodium propionate to the RCA medium. As propionate is a precursor for the synthesis of the higher JH homologs (JH I and II) (section 1.2.2) which are released from the CA of adult female lepidopterans (Baker, 1990), it was thought that JH synthesis may have been enhanced by the addition of propionate into the reaction media. However, CA from 66 hour old virgin female *H. armigera* released similar amounts in the absence (5.1 ± 0.54 pmoles JH/hr/CA) or presence (4.46 ± 0.76 pmoles) of 61μM propionate (n=6) in the RCA medium. Nevertheless, sodium propionate was

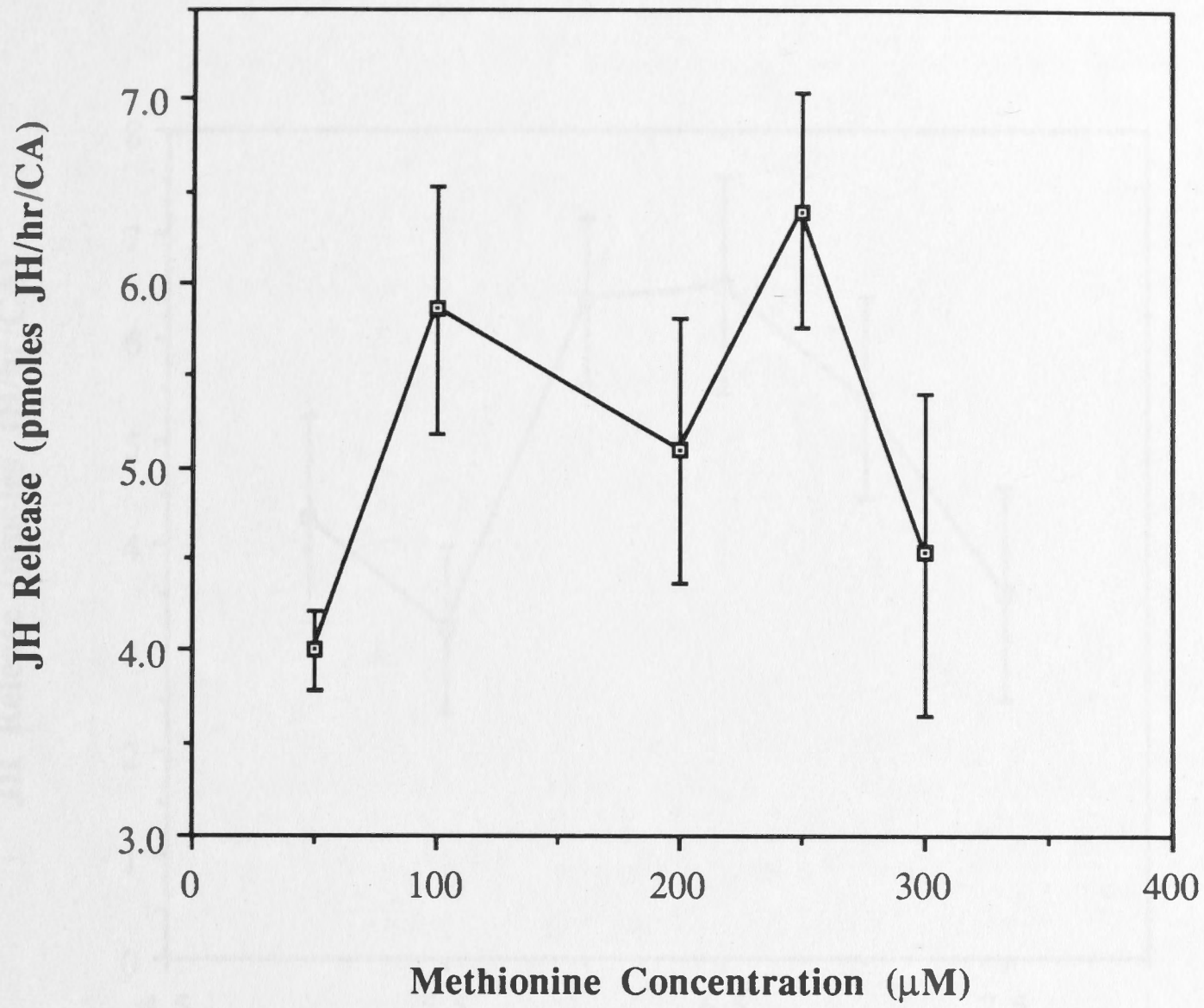


Figure 2.2; Effect of increasing L-methionine concentration on JH release by CA of 48-72 hour old virgin adult female *H. armigera*. Values are expressed as means ± SEM of 6 replicates.

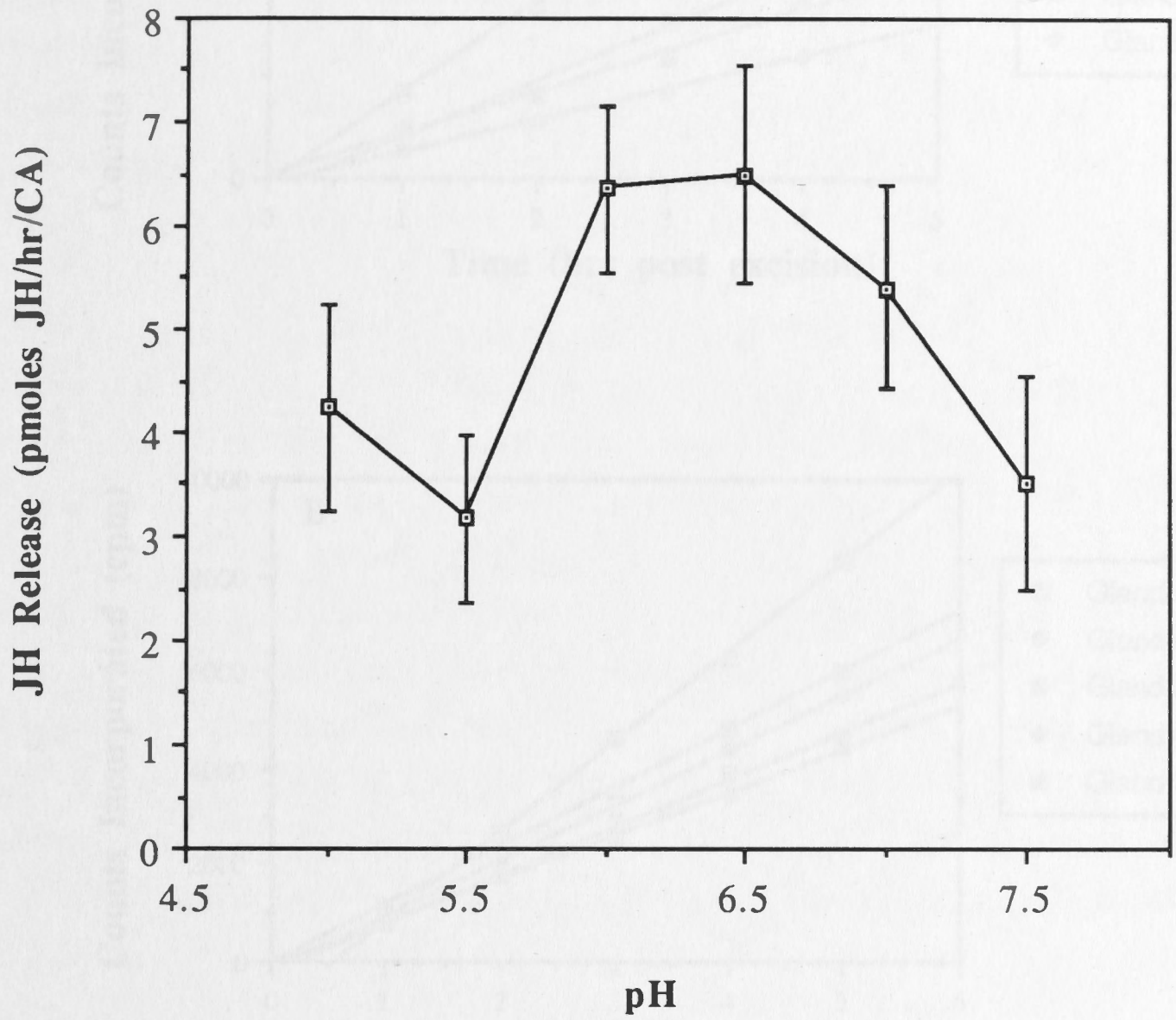
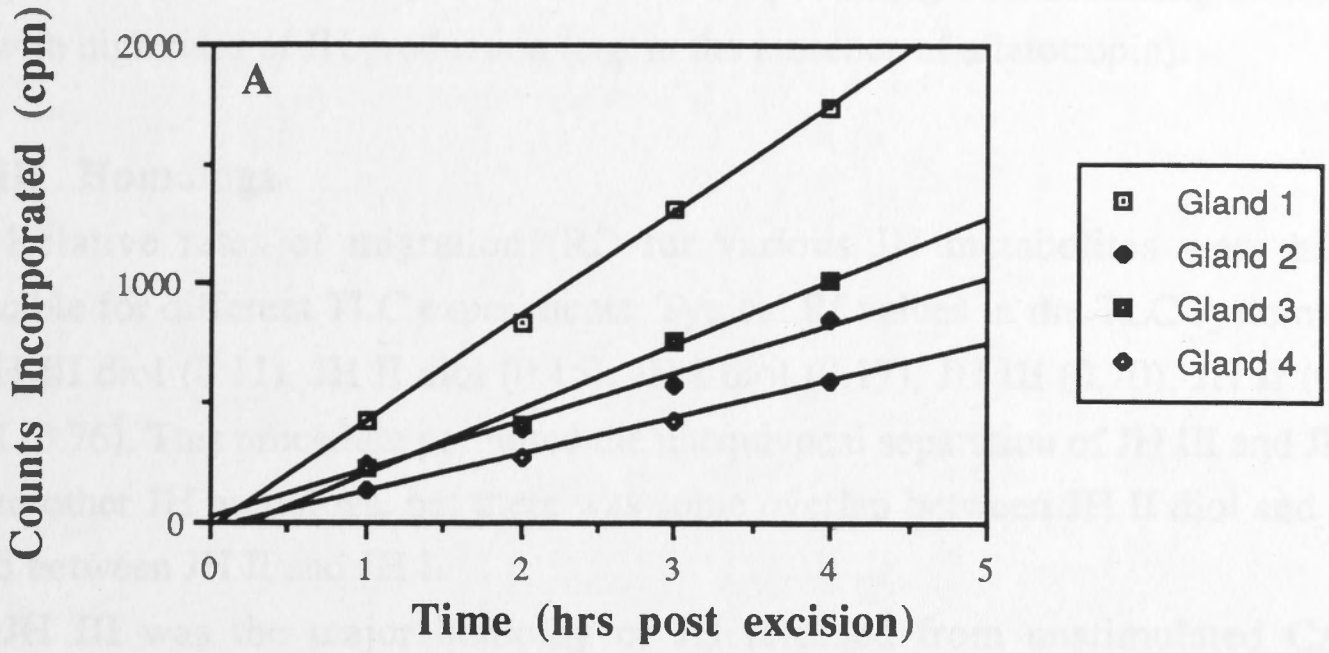


Figure 2.3; Effect of incubation medium pH on JH release from CA of 48-72 hour old virgin adult female *H. armigera*. Values are expressed as means ± SEM of 6-7 replicates.



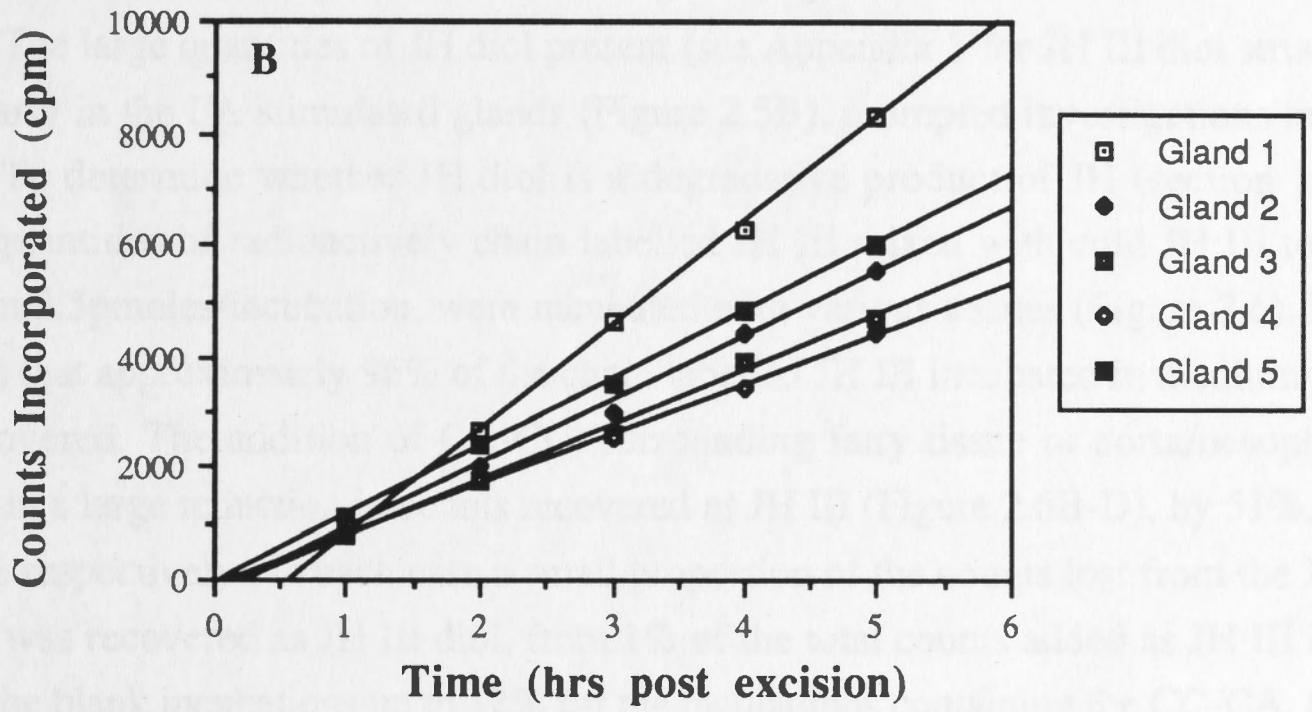


Figure 2.4; The cumulative incorporation of the labelled methyl group from L-[methyl-^{3}H]-methionine into JH, released from CA of four individual 0-12 hour old virgin adult female *H. armigera* (Panel A) and CA from five individual 48-72 hour old virgin adult female *H. armigera* (Panel B). Lines are the simple linear regression of the data points; R^2 = 0.982 to 0.999 for labelled JH release by CA from 0-12 hour old moths and 0.995 to 1 for the older moths.

routinely included in the RCA medium to avoid the possibility of it becoming limiting in glands with high rates of JH production (e.g. in the presence of allatotropin).

2.3.6 JH Homologs

Relative rates of migration (Rf) for various JH metabolites were highly reproducible for different TLC experiments. Typical Rf values in the TLC system used were; JH III diol (0.11), JH II diol (0.15), JH I diol (0.17), JH III (0.70), JH II (0.74) and JH I (0.76). This procedure permitted the unequivocal separation of JH III and JH III diol from other JH homologs, but there was some overlap between JH II diol and JH I diol, and between JH II and JH I.

JH III was the major homolog of JH released from unstimulated CA of *H. armigera* (Figure 2.5A), although some JH II, JH I, JH III diol, JH II diol and JH I diol was also released. Addition of FA resulted in a large increase in the release of JH III and JH III diol from the CA of female *H. armigera* (Figure 2.5B).

The large quantities of JH diol present (see Appendix 3 for JH III diol structure) particularly in the FA stimulated glands (Figure 2.5B), prompted investigations into its source. To determine whether JH diol is a degradative product of JH (section 1.2.4), known quantities of radioactively chain labelled JH III mixed with cold JH III to give 3500cpm/3.5pmoles/incubation, were incubated with various tissues (Figure 2.6). Panel A shows that approximately 96% of the chain labelled JH III incubated in medium alone was recovered. The addition of CA-CC, surrounding fatty tissue or aorta/oesophagus resulted in a large reduction in counts recovered as JH III (Figure 2.6B-D), by 51%, 27% and 56% respectively. In each case a small proportion of the counts lost from the JH III fraction was recovered as JH III diol, from 1% of the total counts added as JH III in the case of the blank incubations up to 12% for the incubations containing the CC-CA, but in no case was the loss of JH III totally accounted for.

In an attempt to explain the loss of labelled JH III, six CA-CC complexes, together with associated tissue normally present (section 2.2.2), were individually incubated with chain labelled JH III mixed with cold JH III to give 5000cpm/3.5pmoles/incubation. Both the aqueous and organic phases were extracted, with 58 ± 3.5% of the counts added being retrieved from the organic phase where JH and JH diol partition, 31.1 ± 2.5% of the counts added being retrieved from the aqueous phase, leaving about 12% of the counts unaccounted for.

2.3.7 Developmental Profile of JH Release in Adult Female *H. armigera*

Developmental profiles were constructed for the *in vitro* release of JH by virgin adult female *H. armigera* CA from the AN and SUS strains (Figure 2.7). The CA from the SUS strain released significantly higher amounts of JH at all ages tested. For both

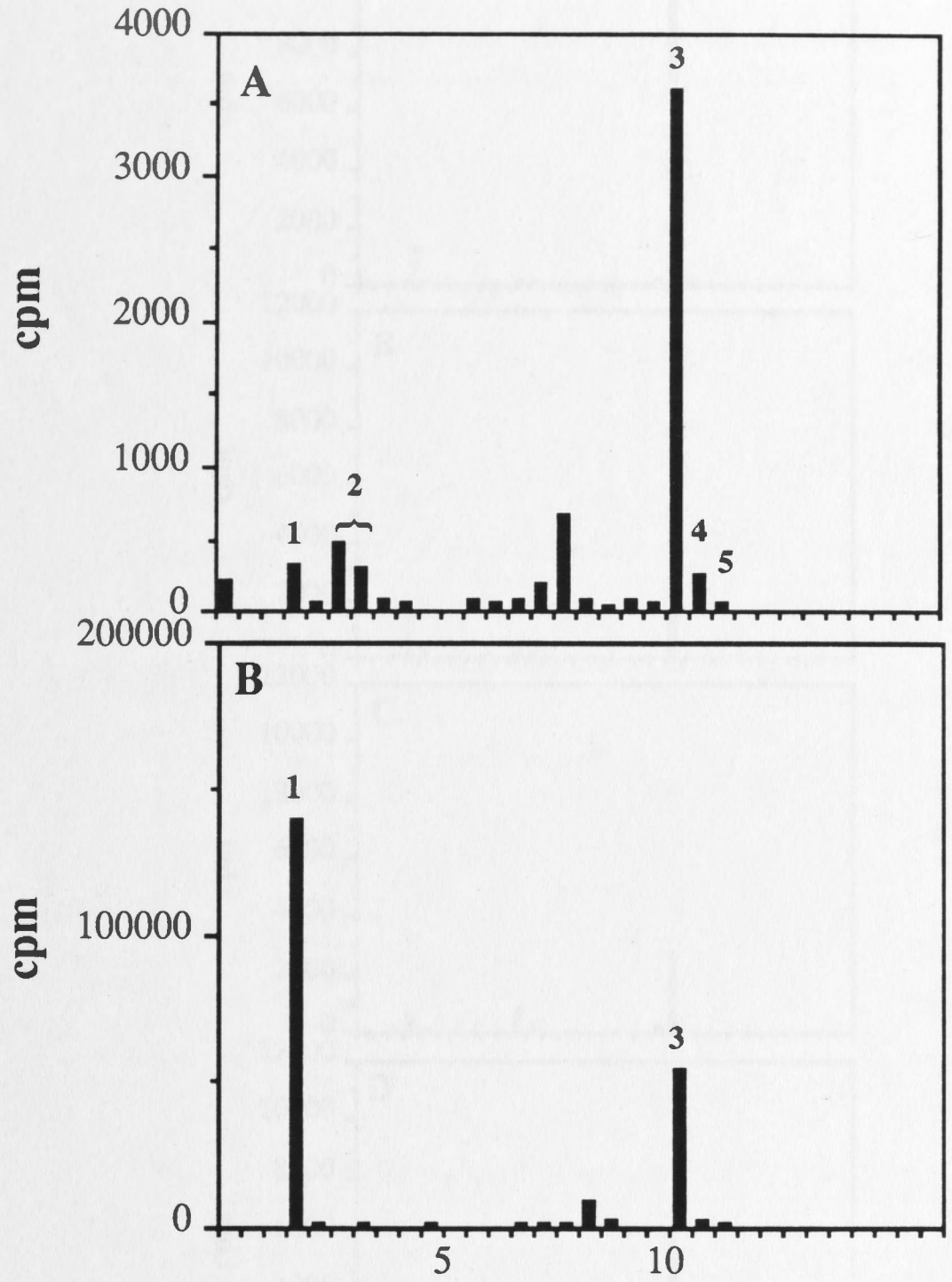


Figure 2.5; TLC radiochromatogram of the products released from the CA of 24-48 hour old virgin adult female *H. armigera* in standard assay medium (Panel A) and standard medium containing 20μM FA (Panel B). Data are the combined products of six glands. Key to identified products; 1=JH III diol, 2=JH II and/or JH I diol, 3=JH III, 4=predominantly JH II and 5=predominantly JH I. Note that the y-axis scales are different for the two radiochromatograms.

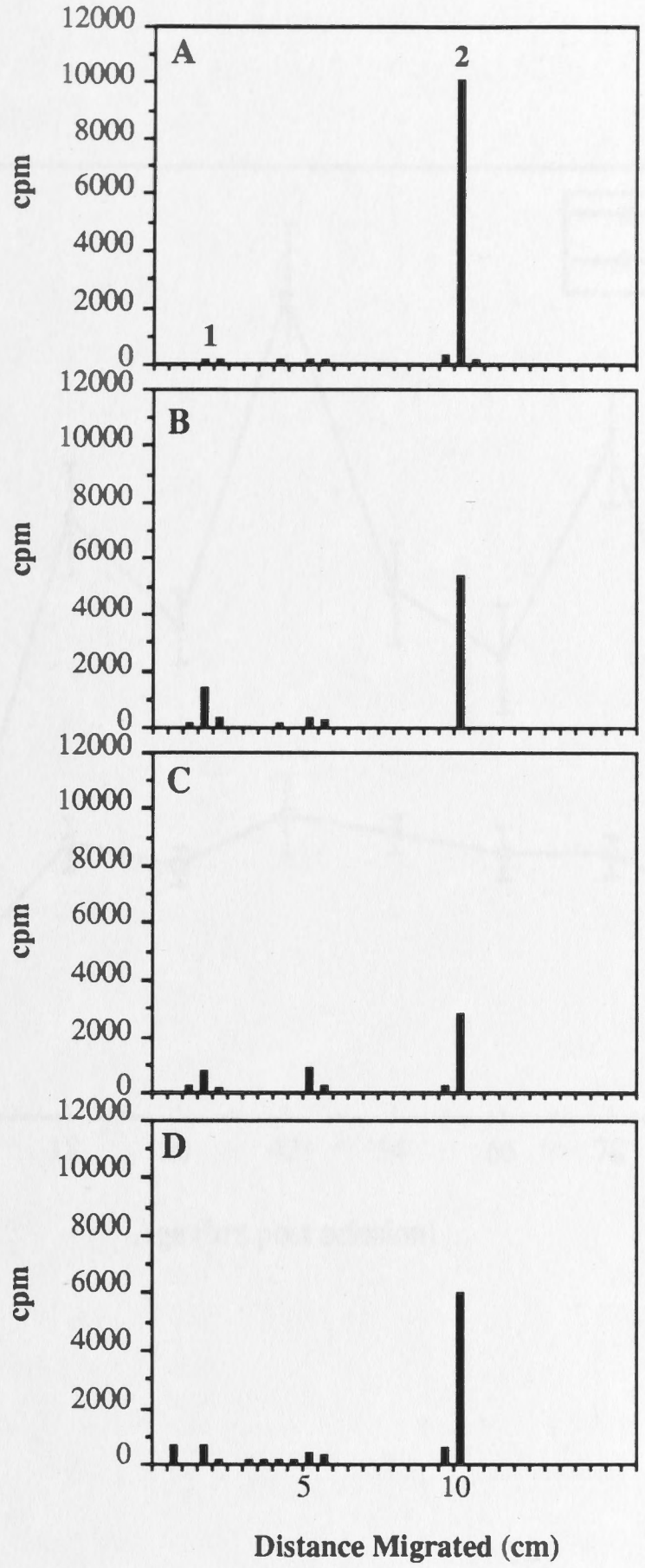


Figure 2.6; TLC radiochromatogram of the radioactivity recovered after the incubation of chain labelled JH III with medium alone (Panel A), CA-CC (Panel B), fat tissue (Panel C) and aorta and oesophagus (Panel D) from virgin adult female *H. armigera* (total of three independent incubations). Key to identified products; 1=JH III diol, 2=JH III.

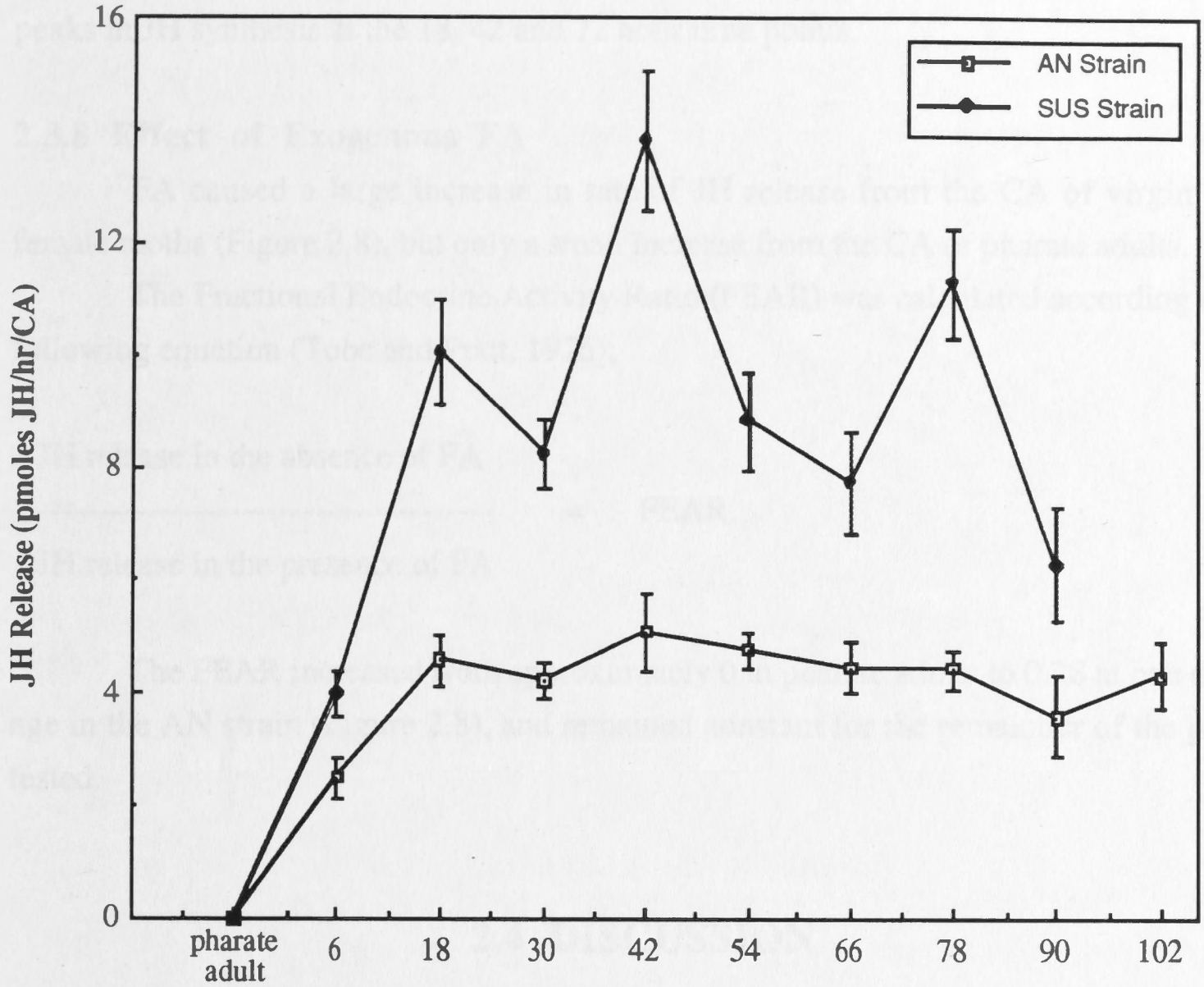


Figure 2.7; Developmental profile of JH released *in vitro* from the CA of virgin adult female *H. armigera* strains AN and SUS. Values are expressed as means ± SEM of 6-27 replicates.

strains, JH synthesis appeared to be initiated about the time of eclosion and increased during the first day of adult life. For the period tested the AN strain showed a constant rate of JH release from this point onwards. In contrast the SUS strain had three apparent peaks in JH synthesis at the 18, 42 and 72 hour time points.

2.3.8 Effect of Exogenous FA

FA caused a large increase in rate of JH release from the CA of virgin adult female moths (Figure 2.8), but only a small increase from the CA of pharate adults.

The Fractional Endocrine Activity Ratio (FEAR) was calculated according to the following equation (Tobe and Pratt, 1976);

$$\frac{\text{JH release in the absence of FA}}{\text{JH release in the presence of FA}} = \text{FEAR.}$$

The FEAR increased from approximately 0 in pharate adults to 0.28 at one day of age in the AN strain (Figure 2.8), and remained constant for the remainder of the period tested.

2.4 DISCUSSION

2.4.1 Hormonal Control of Egg Maturation

Two lines of evidence suggest that *H. armigera* belong to the group of lepidopterans which depend on JH to mature eggs during adulthood (see section 1.1.3.2.3). Firstly, *H. armigera* do not possess any mature eggs in the ovarioles at the time of eclosion, with basal oocyte diameter increasing dramatically over the first two days of adult female life (Table 2.1 and Figure 2.1). Secondly, an essential role of JH for egg production has been established by the ability of JH III and methoprene treatments to restore oocyte maturation in decapitated adult female *H. armigera* (Table 2.1). *H. armigera* can therefore be added to the extensive list of lepidopteran (group II, Table 1.1) and other insect species (Engelmann, 1983) that require JH for egg maturation.

The increase in the number of eggs matured when the amount of the JH analog, methoprene, injected into decapitated females was increased from 1 to 10μg (Table 2.1), points to a dose dependent gonadotropic effect of JH. This has been observed in both lepidopteran (*M. sexta*, Nijhout and Riddiford, 1974; *H. zea*, Satyanarayana *et al.*, 1991; *He. virescens*, Ramaswamy and Cohen, 1991) and non-lepidopteran (*N. cinerea*, Barth and Sroka, 1975; *L. migratoria*, Chen *et al.*, 1976; *D. punctata*, Tobe and Stay, 1979)

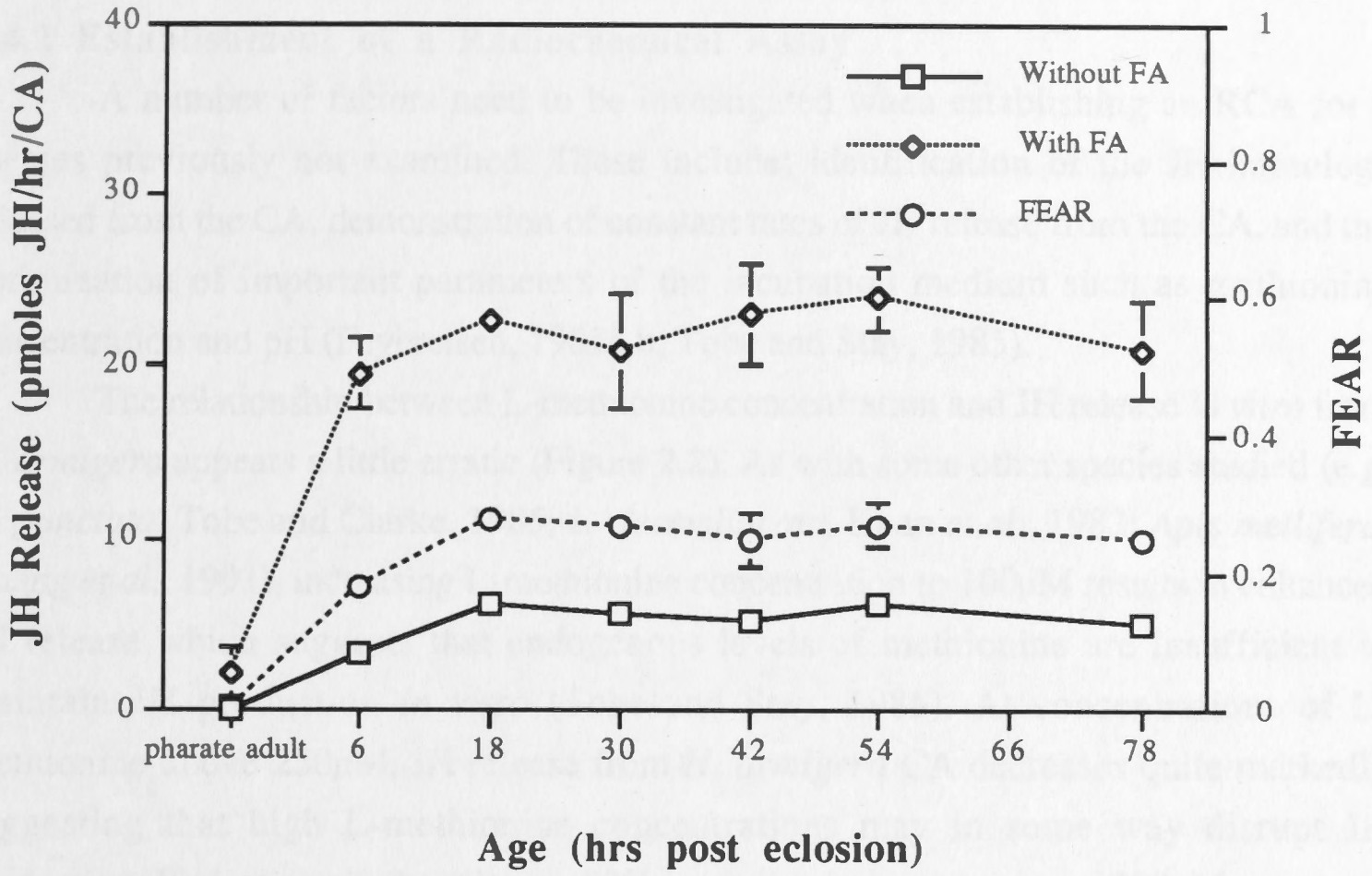


Figure 2.8; Developmental profile of basal JH release and FA stimulated JH release by CA from virgin adult female *H. armigera* of the AN strain. The data collected was used to determine the Fractional Endocrine Activity Ratio (FEAR) of these glands over the period tested. Values are expressed as means ± SEM of 6 replicates.

insect species. The finding that similar quantities of JH and methoprene have significantly different effects on egg production (Table 2.1) is probably attributable to the higher stability of methoprene in the hemolymph, as reported for *M. sexta* larvae (Hatakoshi *et al.*, 1987).

2.4.2 Establishment of a Radiochemical Assay

A number of factors need to be investigated when establishing an RCA for a species previously not examined. These include; identification of the JH homologs released from the CA, demonstration of constant rates of JH release from the CA, and the optimization of important parameters of the incubation medium such as methionine concentration and pH (Feyereisen, 1985a,b; Tobe and Stay, 1985).

The relationship between L-methionine concentration and JH release *in vitro* from *H. armigera* appears a little erratic (Figure 2.2). As with some other species studied (e.g. *D. punctata*, Tobe and Clarke, 1985; *L. decemlineata*, Khan *et al.*, 1982; *Apis mellifera*, Huang *et al.*, 1991), increasing L-methionine concentration to 100μM results in enhanced JH release which suggests that endogenous levels of methionine are insufficient to maintain JH production *in vitro* (Tobe and Stay, 1985). At concentrations of L-methionine above 250μM, JH release from *H. armigera* CA decreases quite markedly suggesting that high L-methionine concentrations may in some way disrupt JH production. Reductions in the release of JH *in vitro* at concentrations of 300μM or greater have also been observed from the CA of foraging honey bees (Huang *et al.*, 1991) and adult *L. decemlineata* (Khan *et al.*, 1982). The concentration of methionine (250μM) which gave the highest levels of JH release in *H. armigera* (Figure 2.2) was used for the RCA. This is approximately the same concentration of methionine which gave optimal JH synthesis in CA from *L. decemlineata* (Khan *et al.*, 1982), day 11 adult female *D. punctata* (Tobe and Clarke, 1985) and the noctuid moth, *P. unipuncta* (Cusson *et al.*, 1990).

Optimal pH of the incubation medium has been found to vary markedly between insect species. The CA from adult female *S. gregaria* produce JH at the greatest rate *in vitro* at a pH of 8, with JH production being reduced by 66% at a pH of 6.5 (Tobe and Pratt, 1974). An optimum pH of 7.2 has been reported for *T. molitor* (Weaver *et al.*, 1980), while a pH of 6.5 gave maximal levels of JH release from the CA of the lepidopteran, *M. sexta* (Kramer and Law, 1980), a value that was also optimal for *H. armigera* (Figure 2.3).

The linearity of JH release from *H. armigera* CA over a period of 5 hours has been established for glands of both high and low activity (Figure 2.4A and B). This allows the results from the RCA experiments to be expressed as rates of JH production (Feyereisen, 1985b). This observation is in accordance with findings in several other

species, where linearity of incorporation of label from L-[methyl-^{3}H]-methionine into JH has been noted for at least five hours (Tobe and Pratt, 1974; Tobe and Stay, 1977; Kramer and Law, 1980; Gadot *et al.*, 1989; Smith *et al.*, 1989; Huang *et al.*, 1991). Some exceptions to the above generalization have been reported in highly active CA from adult female *T. molitor* (Weaver *et al.*, 1980) and *S. gregaria* (Pratt *et al.*, 1975) where JH synthesis decreases with time *in vitro*, probably due to the depletion of JH precursors.

The major JH homolog released from the CA of *H. armigera* is JH III (Figure, 2.5A), with JH II being a relatively minor product. This situation is somewhat different to adult females from other Lepidoptera studied, where JH II, or JH II and JH III, are the predominant JH homologs released from the CA, as seen in *M. sexta* (Peter *et al.*, 1976; Ishizaka *et al.*, 1987; Unni *et al.*, 1991), *P. unipuncta* (Cusson *et al.*, 1990) and *H. zea* (Satyanarayana *et al.*, 1991). Although it was difficult to completely resolve JH I and JH II in the TLC system used, no labelled CA products were detected migrating with JH I, suggesting that JH I is a minor product of the *H. armigera* CA *in vitro*. This observation is in accordance with reports from adult female *M. sexta* (Ishizaka *et al.*, 1987; Unni *et al.*, 1991) and adult female *H. zea* (Satyanarayana *et al.*, 1991) where JH I was only observed in trace amounts, although in *P. unipuncta*, 30% of the JH observed was JH I (Cusson *et al.*, 1990). A unique feature of the *H. armigera* CA products is the presence of JH diols, which are common degradative products of JH (section 1.2.4) and have not been reported to be released from the CA of any other lepidopteran species.

As discussed in Chapter 1 (section 1.2.2) specific roles of JH homologs, if any, in lepidopterans are yet to be elucidated. Until they are, it is difficult to suggest the biological significance of the JH homolog ratios observed in adult female *H. armigera*. As JH III is the dominant JH homolog in *H. armigera* it might be expected to have a greater gonadotropic effect. However, in the dipteran *Aedes atropalpus*, exogenous JH I and JH II, although they are not present in this species, had significantly greater gonadotropic effects than the endogenous hormone JH III (Kelly *et al.*, 1981). Since JH II and perhaps JH I are produced in similar quantities by *H. armigera* CA *in vitro,* it would be interesting to determine their relative gonadotropic effects in this species. Unfortunately this was not possible in the present study because sufficient quantities of JH I and JH II were not available.

Since FA is an intermediate in the synthesis of JH III (section 1.2.3) it was not surprising to discover a dramatic increase in JH III synthesis when CA from adult female *H. armigera* were incubated in its presence. The unusual feature was that a considerable quantity of JH III diol was also found. In most insect species JH diol is believed to be a degradative product of JH and not an intermediate in its synthesis (Hammock, 1985; Schooley and Baker, 1985). Approximately 96% of chain labelled JH III was recovered from the medium after a four hour incubation in assay medium alone, but on addition of

either the retrocerebral complex, fat tissue or aorta/oesophagus, this was significantly reduced (Figure 2.6, Panels B, C and D respectively). A small portion of this loss could be attributed to conversion of JH III to JH III diol, indicating the presence in all these tissues of epoxide hydrolases, which have been observed in other adult lepidopterans (Slade and Wilkinson, 1974; Wisniewski *et al.*, 1986a,b). However the activity of these putative hydrolases does not account for the high levels of JH III diol extracted from the RCA media taken from CA incubated with FA (Figure 2.5B). Taken together these findings indicate that JH III diol could be released from the CA of *H. armigera.*

Gadot *et al.* (1986 and 1987b) working with *L. migratoria* presented a number of lines of evidence to show that JH III diol was released from the CA of that species at levels roughly equivalent to JH III, although in this case allatotropin and FA stimulated glands preferentially released JH III. It is thus possible that JH diol has a biological function (Gadot *et al.*, 1987b) and can be added to the already long list of JHs released from the CA of lepidopteran insects. Theoretically, JH diol, like JH acid in lepidopterans, could be converted to JH in peripheral tissues (section 1.2.3), although an enzyme capable of converting JH diol to JH is yet to be identified (Casas *et al.*, 1991). Alternative possibilities are that an endogenous hydrolase may act to ensure low levels of JH in the virgin state with this enzymatic activity being removed upon mating, or that high levels of JH diol in FA stimulated glands are the result of degradation by non-specific epoxide hydrolases.

The apparent loss of counts occurring when labelled JH III was incubated with small amounts of tissue was investigated further. Following organic extraction, the aqueous phase was subjected to liquid scintillation spectrometry and found to contain almost a third of the radioactivity added. JH acid (see Appendix 3 for the structure of JH III acid), the product of JH esterase hydrolysis (section 1.2.4), and JH acid-diol partition into the aqueous phase upon extraction with the organic solvents used in this study (Share and Roe, 1988) and are most likely the source of the radioactivity observed in this fraction. JH esterase activity has been noted in adult females of the lepidopterans, *T. ni* (Venkatesh *et al.*, 1988) and *M. sexta* (Casas *et al.*, 1991).

Even after scintillation counting of both the organic and aqueous phases, a significant portion (approximately 12%) of the counts remained unaccounted for. As tissue samples were removed before extraction, the labelled JH III which was not found in either of the phases, may have been sequestered in these tissues. This phenomenon has been noted previously by other workers (Nowock *et al.*, 1976; Couillaud *et al.*, 1985; Share and Roe, 1988).

The above evidence suggests that the values for JH release recorded in this study may have been considerably underestimated, since the recovery of labelled JH is reduced in the presence of the CA and surrounding tissue. As this work is a qualitative study of

the times at which JH is released from the CA of adult female *H. armigera,* and a quantitative analysis of JH release was not undertaken, no attempts to increase the recovery of labelled JH (using factors such as JH esterase inhibitors) were made.

2.4.3 JH Biosynthesis During Egg Production

A strong relationship between egg production and CA activity was observed in *H. armigera*, with both basal oocyte diameter (Figure 2.1) and JH release (Figure 2.7) increasing within the first day of adult life. Mature eggs were found within the ovarioles 24 hours later (Table 2.1). A similar relationship between JH production and basal oocyte diameter has been reported for *M. sexta* (Sroka and Gilbert, 1971; Nijhout and Riddiford, 1974; Sasaki and Riddiford, 1984) and *P. unipuncta* (Cusson *et al.*, 1990). Presumably continued JH synthesis is required for maturation of more eggs and possibly for other JH dependent functions (Appendix 1).

The two strains used in this study, AN and SUS, have been cultured independently because of their respective resistance and susceptibility to pyrethroid insecticides (Daly and Fisk, 1992). There was a surprising difference in the rate of JH release by these two strains (Figure 2.7) and if there is a dose dependent effect of JH on egg production (see section 2.4.1), then the SUS strain might be expected to produce more eggs. Although the fecundity of the AN and SUS strains was not measured in these experiments, it is of interest that Fitt (1984), while studying the biology of pyrethroid resistant and susceptible strains of *H. armigera*, found that susceptible moths produced significantly more eggs than resistant ones. My observation implies that differences in the rate of JH release seen between laboratories working on the same species may, at least in part, result from differences between the inbred strains used. For example the four-fold difference in JH production early in *M. sexta* adult female life noted by Unni *et al.*, (1991) when comparing their results to those of Kramer and Law (1980), may be attributable, at least in part, to a strain effect.

The JH III biosynthetic intermediate, FA, has been used to study the capacity of the CA to synthesize JH, because the final two enzymatic steps leading from FA to JH III are not generally thought to be rate limiting (Tobe and Pratt, 1976; Feyereisen, 1985b; Tobe and Stay, 1985). The rate of JH synthesis by CA from the *H. armigera* AN strain increased approximately five fold in the presence of FA. The activity of the two terminal enzymes does not appear to vary over the period of adult life examined as the rate at which FA can stimulate JH production during this time remains constant (Figure 2.8). However the ability of FA to promote JH synthesis during pharate adulthood is relatively small, suggesting that the activity of at least these two terminal enzymes is being established at this developmental stage (Figure 2.8). A distinct heterogeneity was observed in the data used to calculate the pharate adult FEAR value. Three of the six

glands did not release JH whether stimulated by FA or not. The other three CA, although not spontaneously releasing JH *in vitro,* were able to release up to 2.1 pmoles JH/hr/CA when incubated in RCA medium containing FA. Therefore it could be argued that these latter glands have reached a point in development where the capacity of the CA to produce JH is being re-established following the pupal period. Approximately 6 hours after eclosion, glands have acquired the ability to convert FA to JH III at the same rate as any other adult age tested, although JH synthesis in the absence of FA still appears to be increasing (Figure 2.8). This observation may indicate that other enzymes of the JH biosynthetic pathway are still being established, or that regulatory mechanisms controlling JH release are acting during the first day of adult life to activate the pathway.

The FEAR has been used to express the changes in the rate of JH synthesis in relation to the biosynthetic capacity of the two terminal enzymatic steps (Feyereisen, 1985b). Various values of this ratio have been observed during female reproduction in *D. punctata* (Feyereisen *et al.*, 1981a), *L. migratoria* (Gadot and Applebaum, 1986) and *P. americana* (Ratna *et al.*, 1992). Dramatic changes in FEAR, ranging from 0.003 to 0.75, have been observed in adult female *S. gregaria* (Tobe and Pratt, 1976) where peaks of FEAR value are correlated with high JH production during periods of vitellogenesis. FEAR in *H. armigera* increased during the first day of adult life and thereafter remained constant at a relatively low level indicating that at no time was the pathway being utilized to the potential of the two terminal steps. As mating has been reported to increase egg production in *Heliothis* and *Helicoverpa* species (Hardwick, 1965; Proshold, 1982), it is possible that JH synthesis and FEAR may increase at the time of mating.

2.5 SUMMARY

A gonadotropic role for JH has been demonstrated in virgin adult female *H. armigera*. A RCA to measure the *in vitro* release of JH has been established and validated for this species and JH III has been shown to be the predominant JH homolog released from the CA. FA stimulated CA released large quantities of JH III and JH III diol. Evidence has been presented that JH esterase activity, and possibly JH tissue sequestration, greatly decrease the recovery of JH from the RCA media.

The release of JH from the CA of the *H. armigera* strains AN and SUS was found to vary significantly during adult female life. However in both strains JH release was undetectable from female pharate adult CA, with JH synthesis initiated around the time of adult eclosion. The factor(s) responsible for this activation of JH production is the focus of the next Chapter.

CHAPTER 3

NEUROENDOCRINE CONTROL OF JUVENILE HORMONE PRODUCTION IN VIRGIN FEMALE *Helicoverpa armigera*

CHAPTER 3

3.1 INTRODUCTION

The establishment of an RCA for measuring *in vitro* rates of JH synthesis (Chapter 2) provided a basis for investigation of the neuroendocrine control of JH production in adult female *H. armigera*. The developmental profiles of JH release by both strains of *H. armigera* used in this study indicate that JH production in the adult moth is initiated around the time of eclosion (Figure 2.7). Evidence for the existence of a factor(s) which promotes JH synthesis at eclosion, and possibly maintains JH production throughout adult life, is the focus of this chapter.

Traditionally, experiments involving nerve sectioning *in vivo* and application of brain extracts (BE) *in vitro* have been utilized to determine if neuroendocrine factors regulate CA function in insects (see section 1.3). If such neuroendocrine factors are present in the brain of adult female *H. armigera* they may well share structural similarities with the *M. sexta* allatotropic factor (Mas-AT) which is effective in stimulating JH synthesis from the CA of adult female *He. virescens* (Kataoka *et al.*, 1989). To determine if an allatotropic peptide is present in the brain/retrocerebral complex of virgin adult female *H. armigera*, both the activity of BE and Mas-AT needs to be tested on the CA.

As a number of JH homologs are released from the CA of lepidopterans during development (see section 1.2.1 and 2.4.2), including CA of adult female *H. armigera* (section 2.3.6), it has been hypothesized that neuroendocrine factors which regulate the release of specific homologs of JH may exist (Tobe and Stay, 1985). There is some evidence from larval *M. sexta* that this is in fact the case (section 1.3.5), but in the only experiments to use a known regulatory peptide, the Mas-AT did not preferentially stimulate synthesis of any JH homolog by CA of adult female *M. sexta* (Unni *et al.*, 1991).

3.2 MATERIALS AND METHODS

Most of the experimental methods for this Chapter are the same as for Chapter 2, notably tissue dissection (2.2.2), the RCA (2.2.4) and the techniques for the separation of JH homologs (2.2.6).

3.2.1 Preparation of Brain Extracts

The brain extracts (BE) were prepared by suspending brain/retrocerebral complexes, previously dissected and stored at -80°C, in boiling H_2O (75 brains/250µl) for 20 minutes. The tissues were then homogenized in a microfuge tube with a hand-held homogenizer and the extract was centrifuged at 16 000*g* for 15 minutes. The supernatant was removed and applied to a pre-washed (2mls of methanol followed by 8mls of H_2O) Sep-Pak cartridge, washed with 8 mls of 0.1% trifluoroacetic acid (in H_2O) and the sample eluted with 2mls of 0.1% trifluoroacetic acid in methanol. The sample was dried down in a Hetovac centrifugal desiccator and resuspended in culture medium prior to use. All BE were prepared from a mixture of pharate adult and 0-12 hour old adult moth brain and retrocerebral complexes. Except where indicated, the extracts were made from a mixture of male and female tissues.

3.2.2 Effects of Brain Extracts and Synthetic Mas-AT

The Mas-AT (custom synthesized by Peptide Technology, Sydney) was used at a final concentration of 0.2µM. Experiments utilizing this factor and BE were carried out as described for the FA experiments (section 2.2.4). However in the experiment to test the reversibility of the BE effects (section 3.3.3), glands were transferred to fresh media and incubated for a further one and a half hours following the incubation in the presence of BE.

The experimental design for measuring changes in sensitivity of the CA to Mas-AT during *H. armigera* adult female development (section 3.3.4) was as follows. In experiment 1 (section 3.3.4) work was commenced at 0830. Thus the moths, with a median age of 6 hours, had eclosed overnight in the predominantly dark phase (2030 to 0830, see section 2.2.1), whereas moths with a median age of 18 hours had eclosed in the all light phase (0830 to 2030 of the previous day), moths 30 hours old had eclosed in the predominantly dark phase before that, and so on. As a consequence, although all the moths were exposed to the predominantly dark phase immediately prior to assay, moths 6, 30, 54, and 78 hours of age had eclosed in the predominantly dark phase while those in the 18, 42, 66 and 90 hour age groups had eclosed in the all light phase.

In experiment 2 (section 3.3.4) all work was commenced at 2030 (start of the dark phase) with all moths coming out of the light phase when they were assayed and the thus the photoperiods were reversed from experiment 1. Thus the 6 hour time point represents moths which eclosed in the all light phase, the 18 hour time point those that eclosed the previous night in the predominantly dark phase and so on.

3.3 RESULTS

3.3.1 Evidence for Neuroendocrine Regulation of JH Synthesis

BE prepared from *H. armigera* of mixed sexes increased JH synthesis by the CA of 0-12 hour old virgin adult female *H. armigera* in a dose dependent manner (Figure 3.1). Extracts prepared from single sex brain samples stimulated JH synthesis by CA from 0-12 hour old virgin adult female *H. armigera,* 6.89 ± 1.1 fold for female BE (n=9) and 2.79 ± 0.34 fold for male BE (n=7) compared to basal release rates.

Exposure of the CA from 0-12 hour old virgin female *H. armigera* to 0.2μM Mas-AT resulted in a 2.33 ± 0.23 fold stimulation (n=17) of JH release.

3.3.2 Reversibility of the Effects of Brain Extracts on JH Synthesis

The effect of BE (used at 4 brain equivalents per assay) was readily reversible *in vitro* (Figure 3.2). A three fold increase in JH production was observed after addition of the BE, however rates of JH release were rapidly restored to basal levels when the glands were transferred to fresh medium.

3.3.3 Effects of BE and Mas-AT on JH Homolog Ratios

Comparison of the radiochromatograms from BE and Mas-AT stimulated CA (Figure 3.3B and C) to that from unstimulated glands (Figure 3.3A) shows that BE and Mas-AT stimulate the release of all JH homologs observed in the unstimulated CA. The relative quantities of JH homologs released from BE and Mas-AT stimulated glands were the same (Figure 3.3B and C, respectively), including the release of JH III diol and JH II and/or JH I diol.

3.3.4 Changes in Sensitivity of CA to Mas-AT during *H. armigera* Female Reproductive Development

A profile of the sensitivity of CA from adult females of the SUS strain to the Mas-AT peptide showed a cyclic change, with a 24 hour periodicity (Figure 3.4, Experiment 1). Glands taken from females tested 6, 30, 54, and 78 hours after adult eclosion displayed a significantly greater sensitivity to Mas-AT than those from 18, 42, 66 and 90 hour old adult female *H. armigera*. In each case the CA with the highest sensitivity to Mas-AT were obtained from moths which had eclosed in the predominantly dark phase of the photoperiod (between 2030 and 0830).

To investigate this phenomenon further a replicate experiment was carried out 12 hours out of phase with the first, so that the 6, 30, 54 and 78 hour time points represented moths which had eclosed in the all light phase (between 0830 and 2030). A similar pattern in CA sensitivity to the Mas-AT was found during the first two days of

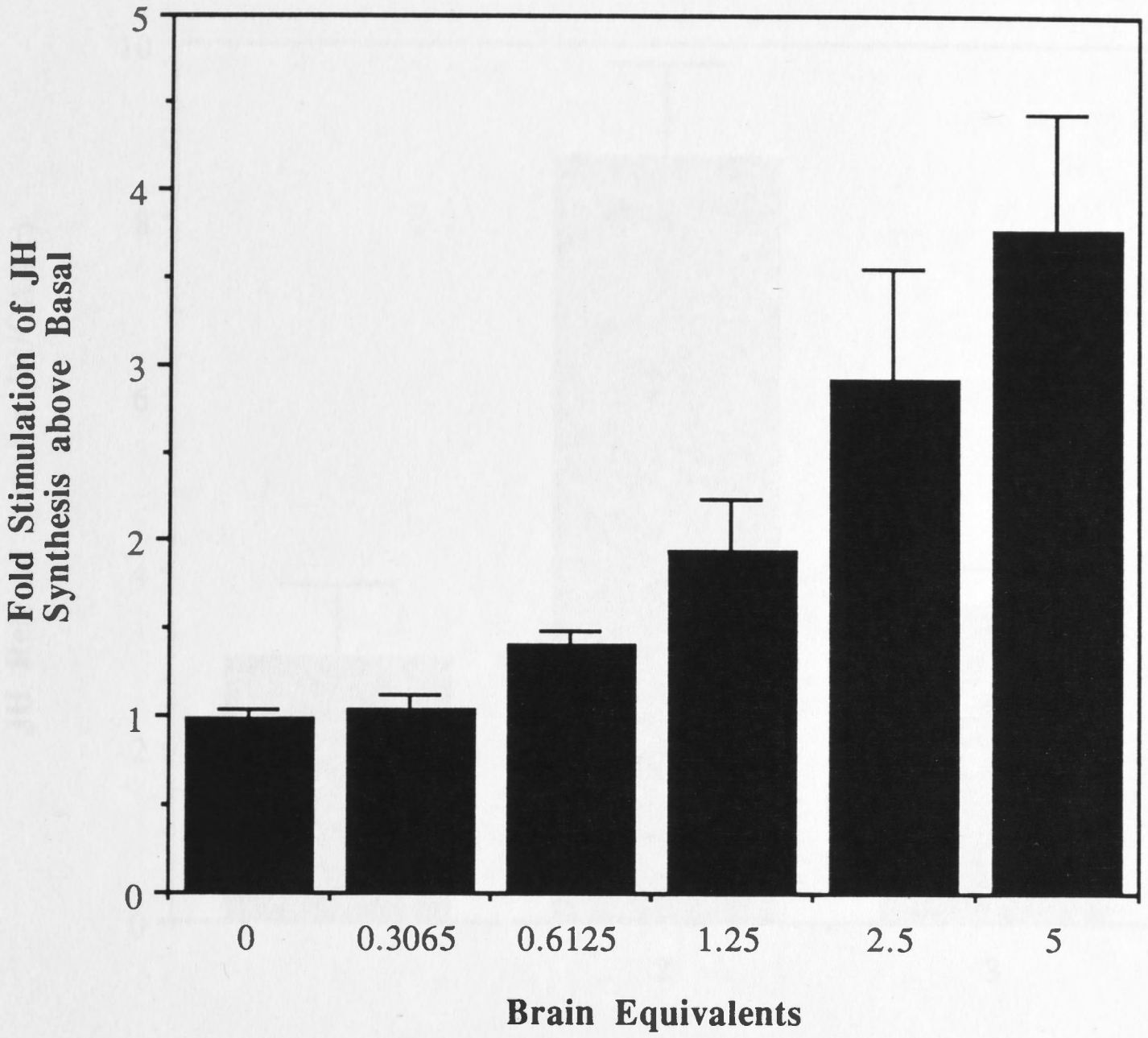


Figure 3.1; Rates of JH release from the CA of 0-12 hour old adult virgin female *H. armigera* in response to varying amounts of BE. Values are expressed as means ± SEM for six replicates.

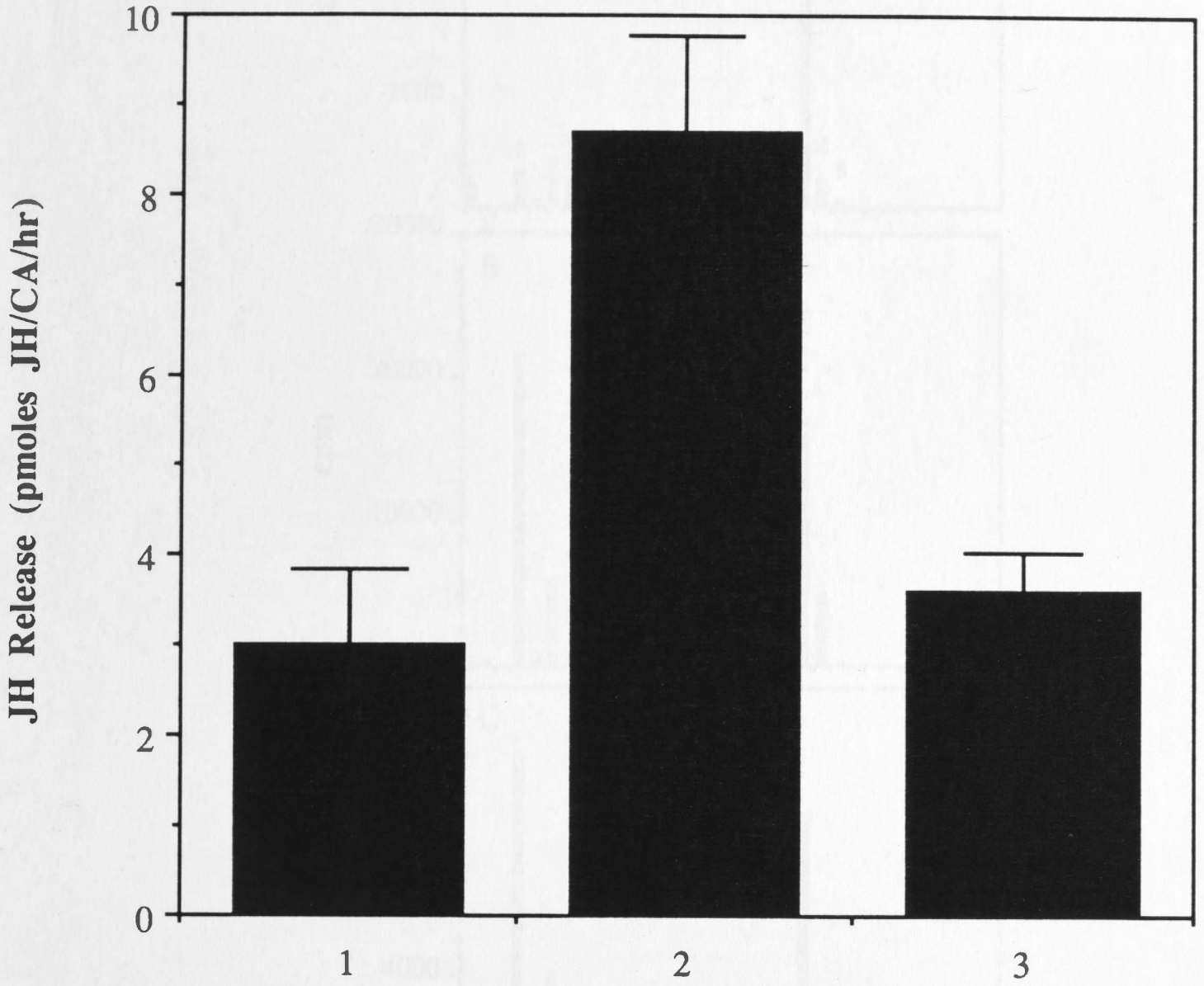


Figure 3.2; Reversibility of the allatotropic effects of BE on JH release from the CA of 0-12 hour old adult virgin female *H. armigera*. Treatment 1 shows the basal level of JH release, treatment 2 is JH release from the same glands in the presence of extracts and treatment 3 is JH release from the same glands after they were transferred to fresh medium without BE. Values are expressed as means ± SEM of 6 replicates.

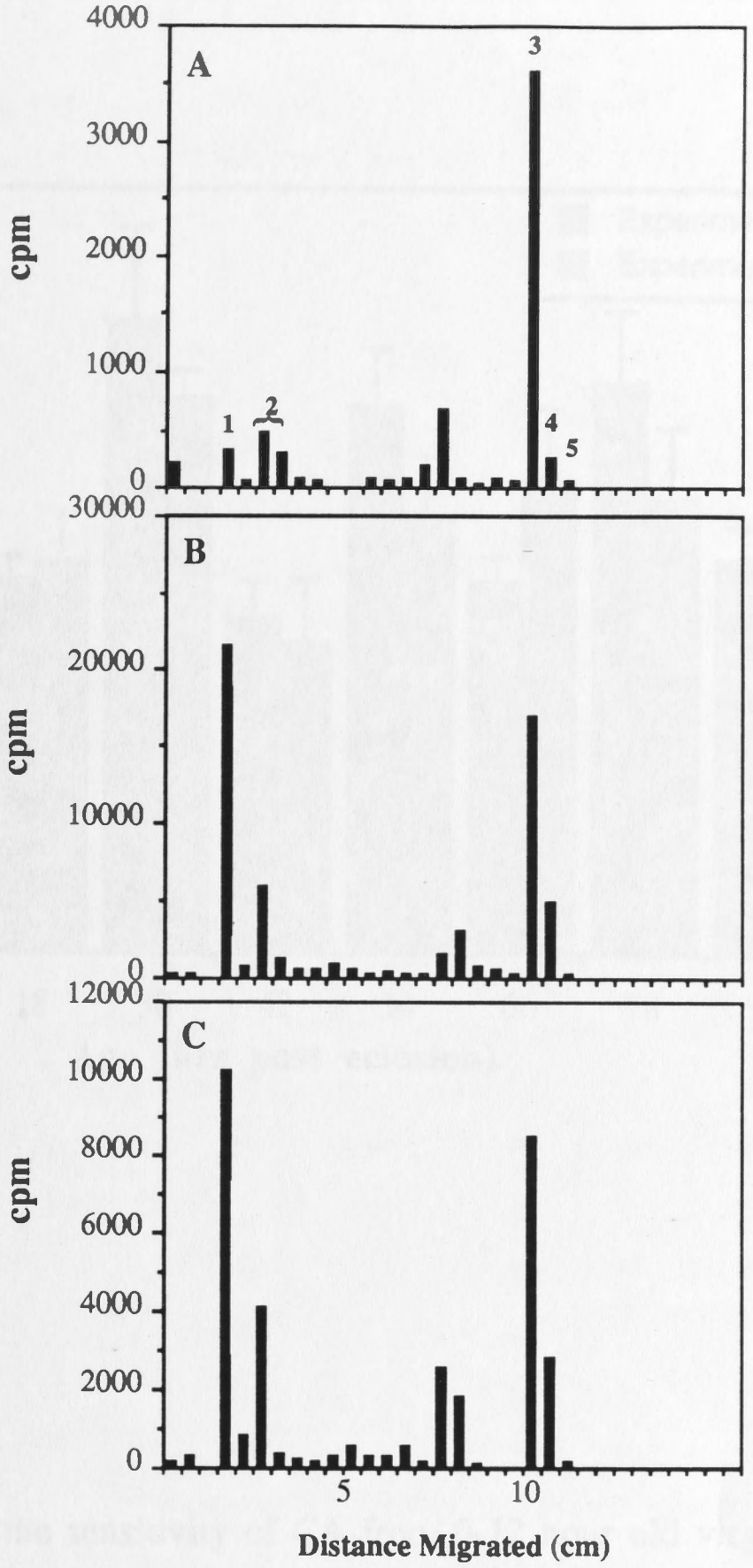


Figure 3.3; TLC radiochromatogram of the products released from CA of 24-48 hour old virgin adult female *H. armigera* of the AN strain in the presence of brain extracts (Panel B) or Mas-AT (Panel C). Figure 2.5A, which shows the homologs of JH released from unstimulated CA, has been repeated as Figure 3.3A for ease of comparison. Data represents the total products of six glands. Key to identified products; 1=JH III diol, 2=JH II and/or JH I diol, 3=JH III, 4=predominantly JH II, 5=predominantly JH I. Note that the y-axis scales are different in each of the radiochromatograms.

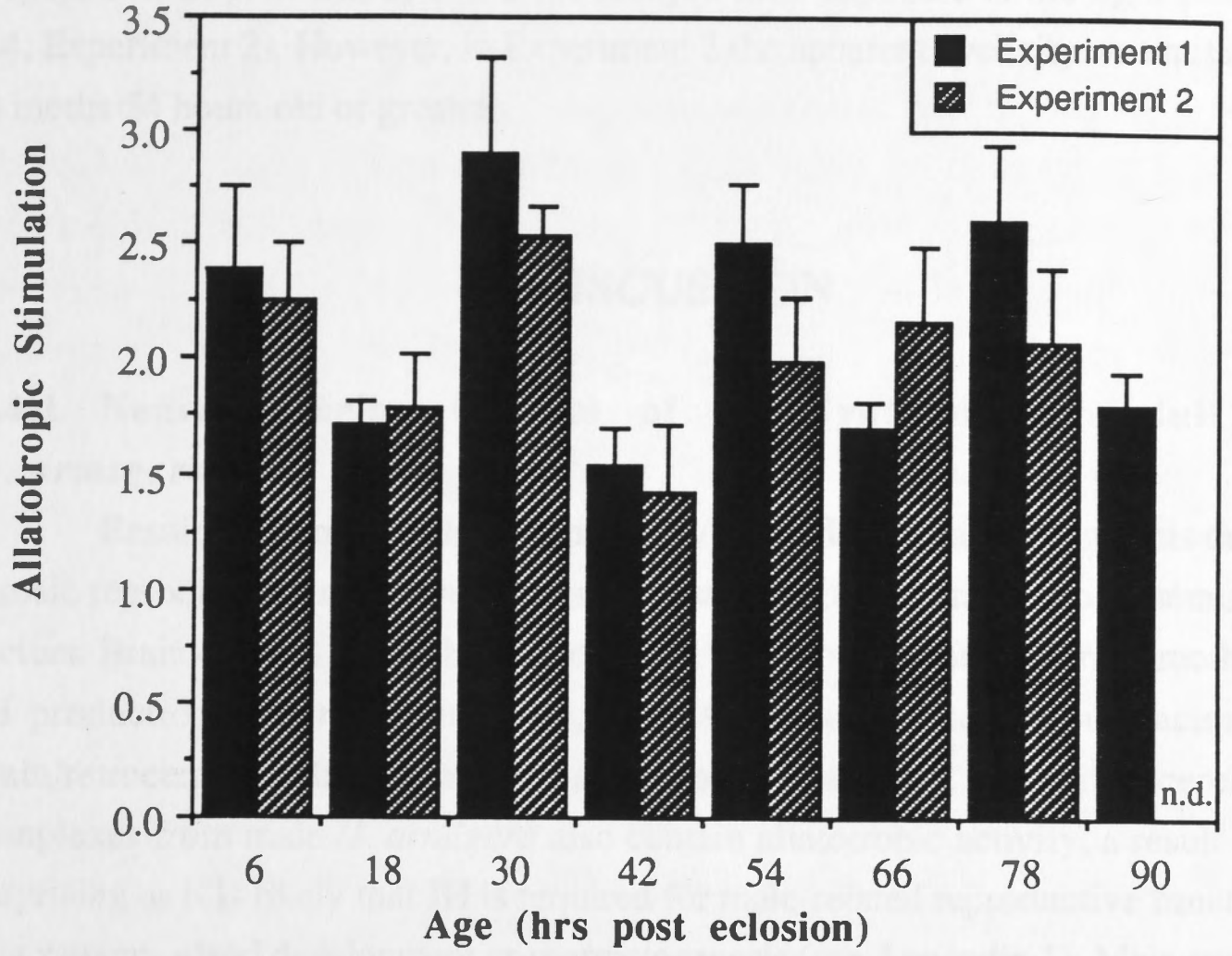


Figure 3.4; Profile of the sensitivity of CA from 0-12 hour old virgin adult female *H. armigera* from the SUS strain to Mas-AT. In Experiment 1, glands used at the 6, 30, 54 and 78 hour time points were taken from moths that had eclosed in the predominantly dark phase. Experiment 2 was 12 hours out of phase with Experiment 1, so that glands used at the 6, 30, 54 and 78 hour time points were taken from moths that had eclosed in the all light phase and those CA used at the 18, 42 and 66 hour time points were taken from moths that eclosed in the predominantly dark phase. Values are expressed as means ± SEM of 6-10 replicates. n.d.=not determined.

adult life for those moths assayed after being exposed to the predominantly dark phase (Figure 3.4, Experiment 1) and those assayed after exposure to the light phase (Figure 3.4, Experiment 2). However, in Experiment 2 the apparent cyclicity was not maintained in moths 54 hours old or greater.

DISCUSSION

3.4.1 Neuroendocrine Control of JH Synthesis in Adult Female *H. armigera*

Results presented in this Chapter provide evidence that JH synthesis during adult female reproduction in *H. armigera* is at least partially dependent upon neuroendocrine factors. Brain extracts from pharate adult and 0-12 hour old adult female moths increase JH production *in vitro* (Figure 3.1), indicating that an endogenous factor(s) in the brain/retrocerebral gland complex has allatotropic activity. Brain/retrocerebral gland complexes from male *H. armigera* also contain allatotropic activity, a result that is not surprising as it is likely that JH is required for male-related reproductive functions, such as accessory gland development or spermatogenesis (see Appendix 1). Male extracts were not as active as those from females suggesting that levels of allatotropin are not as high in males as they are in females.

The Mas-AT peptide stimulates JH release from the CA of adult female *H. armigera*, as was previously reported for another noctuid moth *He. virescens* (Kataoka *et al.*, 1989). This result implies the presence in the *H. armigera* CA of a functional receptor-mediated signalling pathway coupled to JH synthesis, that recognizes the Mas-AT peptide. The occurrence of such a pathway suggests that there may be an allatotropic peptide homologous to Mas-AT in *H. armigera*.

The Mas-AT peptide does not appear to be as active as the endogenous factor(s). A number of reasons can be suggested for this observation. There may be sequence differences between the peptides, or a number of allatotropic factors with additive effects may be present in the *H. armigera* extracts. These two possibilities are unlikely in the light of the gene cloning work (see section 6.3 for discussion). It is perhaps more probable that the apparent low activity of the synthetic peptide is an experimental artifact. Frequent, repeated freezing and thawing of the peptide stock during experiments leading to the establishment of the RCA may have resulted in oxidation of either or both methionines in Mas-AT (Figure 1.6), thus reducing its potency.

The allatotropic factor(s) present in the *H. armigera* BE has a dose dependent and readily reversible effect on JH synthesis *in vitro*. These two characteristics have been noted for many JH regulatory factors studied to date, including allatotropins from adult

female *L. migratoria* (Gadot and Applebaum, 1985), and adult female *M. sexta* (Mas-AT) (Unni *et al.*, 1991) as well as allatostatins/allatinhibins from adult female *D. punctata* (Rankin and Stay, 1987; Woodhead *et al.*, 1989), 4th instar larvae of *M. sexta* (Granger and Janzen, 1986) and 3rd instar larvae of *Sarcophaga bullata* and *D. melanogaster* (Richard *et al.*, 1990). This dose dependence and reversibility of effect, combined with properties observed during preparation of BE (i.e. methanol solubility, resistance to boiling and its ability to be fractionated by reverse phase chromatography) are all consistent with the allatotropic factor(s) in the BE being a peptide.

3.4.2 Neuroendocrine Regulation of JH Homolog Ratios

BE from *H. armigera* and Mas-AT do not appear to preferentially stimulate the release of specific JH homologs from the CA of adult female *H. armigera* (Figure 3.4B and C respectively). This observation is in accordance with that of Unni and co-workers (1991) who found that Mas-AT increased the production of all three JH homologs which are normally released from unstimulated CA. Therefore, in *M. sexta* and *H. armigera* the allatotropic factor(s) which stimulates JH synthesis during adult female reproductive development does not play any obvious role in governing JH homolog ratios.

3.4.3 Sensitivity of *H. armigera* CA to Mas-AT during Adult Female Reproductive Development

The Mas-AT sensitivity experiments indicate that a circadian control of receptivity of the *H. armigera* CA to allatotropin may be acting for at least the first two days after eclosion. This is clearly driven by age and not photoperiod since the peaks in JH production in response to Mas-AT occur at 6, 30 and 54 hours after eclosion, no matter whether the moths eclosed in the morning (Figure 3.4, Experiment 1) or in the evening (Figure 3.4, Experiment 2). If a similar pattern of response to allatotropin occurs *in vivo*, this may mean that JH synthesis *in vivo* is pulsatile. A number of other reports imply that JH may be released in pulses (Feyereisen, 1985b; Khan *et al.*, 1982; Ruegg *et al.*, 1986; Unni *et al.*, 1991), although no diurnal rhythm was observed for activity of the CA of *L. decemlineata* (Khan *et al.*, 1982), *Teleogryllus commodus* (Ruegg *et al.*, 1986) and *D. punctata* (unpublished data cited by Cusson *et al.*, 1990).

The periods of higher sensitivity of the CA, from 6 and 30 hour old moths (Figure 3.4, Experiments 1 and 2), were followed 12 hours later by peaks in JH synthesis at 18 and 42 hours of age as seen in the developmental profile of basal hormone release for the SUS strain (Figure 2.7). This suggests that the fluctuations in JH synthesis may, at least in part, be controlled by the sensitivity of the CA to allatotropins. Similar experiments with the AN strain have not been done, but may be informative since it showed no age related changes in the rates of JH release *in vitro*.

In the field, *H. armigera* eclose predominantly at night (Riley *et al.*, 1992). However, in the inbred laboratory strain of *H. armigera* used in this study approximately 40% of the moths eclose in the light phase (personal observation). Therefore, in the field, age is correlated with photoperiod but this correlation is much weaker in the laboratory environment. In experiment 2 (Figure 3.4), where moths at the 6, 30, 54 and 78 hour time points eclosed in the light phase, cyclical sensitivity of the CA to Mas-AT breaks down after two days. Thus, it is possible that the circadian regulation is initially age dependent, with photoperiod being involved in the maintainance of this rhythm, but this will be difficult to test with laboratory populations.

The circadian regulation of many aspects of the insect life cycle is well documented (Page, 1985) and is often controlled by photoperiod (Beck, 1980; Page, 1985). One example of the circadian control of reproductive behaviour in adult female noctuid moths is that of pheromone release (Sower *et al.*, 1970), which is initiated by JH and governed by the pheromone biosynthesis-activating neuropeptide (Cusson and McNeil, 1989). Males of four noctuid species (*Autographa californica*, *He. virescens*, *Spodoptera exigua* and *T. ni*) exhibit cyclic, diurnal changes in their responsiveness to sex pheromones from females which can be disrupted by manipulation of the photoperiod (Shorey and Gaston, 1965).

It must be stressed that the apparent circadian sensitivity of *H. armigera* CA to Mas-AT is only a preliminary observation. Further experiments which record the exact age of the moths, the time at which they eclosed and which uses *H. armigera* pupae collected from the field, would probably help in the clarification of the observed cyclicity. There are many questions related to the apparent cyclicity in CA sensitivity to Mas-AT which also need to addressed. For example, are there fluctuations in the number of allatotropin receptors during the day, are periods of low CA sensitivity times when there are large quantities of allatotropin present *in vivo*, is the release of allatotropin from NSC also under circadian regulation, are there fluctuations of JH titre *in vivo* and if so, how are these fluctuations related to JH function?

3.5 SUMMARY

BE prepared from female and male pharate adult and newly eclosed *H. armigera* both contain an allatotropin(s). The effects of BE in stimulating JH release were dose dependent and readily reversible. JH production could also be stimulated by Mas-AT, with this factor and the BE both increasing levels of all the JH homologs previously observed to be released from *H. armigera* CA. Evidence suggests an age dependent circadian control in the sensitivity of the CA to an allatotropin may exist.

The ability of BE and Mas-AT to stimulate JH release from the CA of adult female *H. armigera* raises the possibility that a peptide homologous to Mas-AT is produced in *H. armigera* brains. This is the basis for the allatotropin gene cloning experiments presented in the next Chapter.

CHAPTER 4

PARTIAL CHARACTERIZATION OF THE *Helicoverpa armigera* ALLATOTROPIC FACTOR GENE AND PREPROHORMONE

CHAPTER 4

4.1 INTRODUCTION

Traditionally, neuropeptide characterization has required the accumulation of large quantities of tissue, followed by extraction and multiple fractionation steps, culminating in sequencing of the purified peptide (Schooley *et al.*, 1990). Despite many technological advances in the above process since it was first used to isolate proctolin from *P. americana* (Brown and Starratt, 1975), including the discovery of ion-pairing reagents used in reverse-phase high-performance liquid chromatography and the development of gas-phase sequencing (Holman *et al.*, 1990), considerable effort is still required to characterize neuropeptides (Kelly *et al.*, 1990). For example, the isolation of the *M. sexta* allatotropic factor (Mas-AT) required 10 000 trimmed heads (420g) to obtain 1.5nmoles of the pure peptide (Kataoka *et al.*, 1989).

The ability of the Mas-AT peptide to stimulate JH release from the CA of adult female *H. armigera* (see Chapter 3) opens up the possibility of a molecular genetic approach (Douglass *et al.*, 1984; Holman *et al.*, 1990; Kelly *et al.*, 1990) for the characterization of the homologous peptide from *H. armigera.* As the gene encoding Mas-AT had not been isolated at the onset of my project, a gene cloning strategy using oligonucleotide probes was the most reasonable option. Oligonucleotides are short tracts of single-stranded DNA that can be designed from amino acid sequence data, radioactively labelled and used to detect target sequences (Lathe, 1985). They have been successfully used to obtain the gene encoding the *M. sexta* eclosion hormone (Horodyski *et al.*, 1989) and, during the course of my project, several other insect neuropeptide preprohormone genes were isolated using this approach. These included the pheromone biosynthesis activating hormone gene from *H. zea* (Davis *et al.*, 1992), the *M. sexta* diuretic hormone gene (Digan *et al.*, 1992) and the *B. mori* diapause hormone gene (Sato *et al.*, 1993).

This chapter reports the isolation of genomic clones from *M. sexta*, and genomic and cDNA clones from *H. armigera* that potentially code for Mas-AT and Hea-AT peptides respectively. The partial structure of the Hea-AT gene, the putative partial Hea-AT preprohormone sequence, and partial structure of the putative Mas-AT preprohormone are presented.

4.2 MATERIALS AND METHODS

4.2.1 DNA Isolation

A number of protocols were used to isolate DNA for various applications;

a) Genomic DNA was prepared from *H. armigera* eggs and neonates using the Lifton extraction procedure developed by Blin and Stafford (1976) and from *M. sexta* eggs (a gift of Dr M. Greenstone, Department of Agriculture, U.S.A.) as described by Davis *et al.* (1986).

b) Recombinant phage DNA was isolated using a modified liquid lysis procedure (Appendix 4).

c) Plasmid DNA was obtained using an alkaline lysis method (Maniatis *et al.*, 1982).

d) Single stranded DNA for sequence analysis was prepared using M13KO7 as a helper phage, following the protocol of Vieira and Messing (1987).

4.2.2 Genomic Library Construction

Genomic libraries for *M. sexta* and *H. armigera* were constructed in the phage vector λGEM-11 (Promega) using the procedure devised by Frischauf *et al.* (1983). Approximately 100μg of genomic DNA was partially digested with *Sau 3A* (Sambrook *et al.*, 1989) and size fractionated on a sucrose gradient (Luthe, 1983). DNA in the size range of 15-20kb was selected and ligated to λGEM-11, which was previously digested with *Bam HI* and dephosphorylated (*Sau 3A* and *Bam HI* digestion produce compatible cohesive ends). Recombinant phage DNA was packaged into phage particles using the Promega 'Packagene' system.

An estimated $5x10^5$ independent phage clones were obtained for the *H. armigera* library and $1x10^6$ for the *M. sexta* library. Both libraries were amplified (Maniatis *et al.*, 1982) and stored at -80°C. The respective titres of the amplified libraries for *H. armigera* and *M. sexta* were $4x10^{11}$ and $1x10^{12}$ pfu/ml.

4.2.3 Oligonucleotide Design

A number of different oligonucleotides, synthesized on a Pharmacia Gene Assembler-Plus, were used in various attempts to isolate the allatotropic genes. Oligonucleotides were designed from the Mas-AT peptide sequence given in Figure 4.1. Some of these assumed the existence of a carboxyl terminal glycine residue, predicted to be present in the prohormone sequence to provide the substrate for peptide amidation (section 1.3.2.4). In some instances, to reduce probe degeneracy, the base composition of oligonucleotides was biased according to patterns of codon usage characterized in genes from other lepidopteran species; *B. mori* (Wada *et al.*, 1990) and *M. sexta* (constructed from 13 *M. sexta* gene sequences obtained from GenBank database)

A)

NH_2-	GLY	PHE	LYS	ASN	VAL	GLU	MET	MET	THR	ALA	ARG	GLY	PHE	GLY	-COOH
	GGT 0.38	TTC 0.85	AAG 0.75	AAC 0.75	GTC 0.38	GAG 0.60	ATG 1.00	ATG 1.00	ACC 0.47	GCC 0.40	CGC 0.28	GGT 0.38	TTC 0.85	GGT 0.38	
	GGC 0.33	TTT 0.15	AAA 0.25	AAT 0.25	GTG 0.32	GAA 0.40			ACT 0.25	GCT 0.36	AGA 0.24	GGC 0.33	TTT 0.15	GGC 0.33	
	GGA 0.23				GTT 0.17				ACA 0.15	GCG 0.15	CGT 0.20	GGA 0.23		GGA 0.23	
	GGG 0.06				GTA 0.13				ACG 0.13	GCA 0.09	AGG 0.16	GGG 0.06		GGG 0.06	
											CGA 0.07				
											CGG 0.06				

B)

NH_2-	GLY	PHE	LYS	ASN	VAL	GLU	MET	MET	THR	ALA	ARG	GLY	PHE	GLY	-COOH
	GGT 0.31	TTC 0.83	AAG 0.74	AAC 0.74	GTC 0.31	GAG 0.50	ATG 1.00	ATG 1.00	ACC 0.30	GCC 0.32	CGC 0.15	GGT 0.31	TTC 0.83	GGT 0.31	
	GGC 0.37	TTT 0.17	AAA 0.26	AAT 0.26	GTG 0.24	GAA 0.50			ACT 0.30	GCT 0.32	AGA 0.27	GGC 0.37	TTT 0.17	GGC 0.37	
	GGA 0.26				GTT 0.28				ACA 0.23	GCG 0.18	CGT 0.27	GGA 0.26		GGA 0.26	
	GGG 0.06				GTA 0.17				ACG 0.17	GCA 0.17	AGG 0.22	GGG 0.06		GGG 0.06	
											CGA 0.07				
											CGG 0.02				

Figure 4.1; Peptide sequence of Mas-AT with a C-terminal glycine added as an α-amidation substrate. For each amino acid the codon frequency is given for A) *B. mori* (Wada *et al.*, 1990) and B) *M. sexta*. Masat 1, 2, 4a/c and 4a/c II were derived from *B. mori* codon usage data. Masat 6 and 7 were derived from *M. sexta* codon usage data. Please note that the Arg values in A) and the Ala values in B) do not add up to 1, presumably due to rounding the figures off to two decimal places during their calculation.

(Figure 4.1).

The sequences and details of these oligonucleotides are given below. The number of times a base is presented at a particular position indicates the percentage of oligonucleotides within the pool that contain that base at that position. For example if a G and an A are given, then 50% of the oligonucleotides have a guanine at that position and 50% an adenine; or if there are 4 Cs and one T then 80% of the oligonucleotides have a cytosine at that position and 20% a thymine (as seen below at postions 3 and 24 respectively of Masat 4a). The region of the Mas-AT amino acid sequence from which the individual oligonucleotides were designed is given above the oligonucleotide sequence.

Masat 1; a unique sequence oligonucleotide which, according to the table of codon usage for *B. mori* genes, is most likely to be the 19 3' bases encoding Mas-AT (not including a possible C-terminal glycine).

```
      MetThrAlaArgGlyPhe
5'-GATGACCGCCCGCGGTTTC-3'
```

Masat 2; a unique antisense sequence oligonucleotide which, according to the *B. mori* gene codon usage table, is most likely to be the 18 5' bases of Mas-AT.

```
    GlyPheLysAsnValGlu
3'-CCAAAGTTCTTGCAGCTC-5'
```

Masat 4a/c; two 26 nucleotide, 798 fold degenerate oligonucleotides (designed from *B. mori* codon usage table) which encompasses possible coding sequences of the C-terminal end of Mas-AT (including a C-terminal glycine) used in a equimolar mixture.

Masat 4a:

```
    GluMetMetAlaThrArgGlyPheGl
5'-GAGATGATGACCGCTAGGGGATTCGG-3'
   A     C  C  A  T  C
         T  T     C  C
         G  C        C
         A  A        T
            G
```

Masat 4c:

```
      GluMetMetAlaThrArgGlyPheGl
5'-GAGATGATGACCGCTCGTGGATTCGG-3'
   A        C  C  C  T  C
            T  T     C  C
            G  C        C
            A  A        T
               G
```

Masat 4a/c II; are the same as Masat 4a/c except the three 5' bases are not included, making these oligonucleotides 23 nucleotides in length and 384 fold degenerate.

Masat 6; a 26 nucleotide, 128 fold degenerate oligonucleotide which encodes the N-terminal end of Mas-AT (designed using the *M. sexta* codon usage table).

```
      GlyPheLysAsnValGluMetMetTh
5'-GGGTTCAAGAATGTGGAGATGATGAC-3'
   A     A  C  A  A
   T           T
   C           C
```

Masat 7; a 41 nucleotide, 16 fold degenerate oligonucleotide encompassing the full sequence of Mas-AT (including a C-terminal glycine), designed using the *M. sexta* codon usage table, with inosines (X) incorporated at sites which have a degeneracy greater than two.

```
      GlyPheLysAsnValGluMetMetThrAlaArgGlyPheGl
5'-GGXTTCAAGAATGTXGAGATGATGACXGCXAGXGGXTTCGG-3'
      A  C     A        C
```

4.2.4 Probe Synthesis

4.2.4.1 Oligonucleotide Probes

Oligonucleotide probes were radiolabelled by the transfer of a radioactive phosphate group from γ-[^{32}P]-dATP (Amersham) to the 5' end of the oligonucleotide by the enzyme T4 polynucleotide kinase (Pharmacia), using the procedure described by Sambrook *et al.* (1989).

4.2.4.2 Random Primed Probes

Cloned genomic DNA was radioactively labelled (using α-[^{32}P]-dATP, Amersham) by the synthesis of complementary strands of DNA from 3' OH ends of

random hexanucleotide primers using the Klenow fragment of *Escherichia coli* DNA polymerase I (Feinberg and Vogelstein, 1983 and 1984). The procedure followed was that supplied with the Random Primed Kit (Boehringer Mannheim).

4.2.5 Genomic Library Screening

Cells of the *E. coli* strain LE392 were infected with recombinant phage and plated on 15cm diameter plates at a density of approximately 20 000 plaques per plate. In each screening 80 000 to 100 000 clones were examined. Phage DNA was transferred in duplicate to nitrocellulose filters (Schleicher and Schuell) as described by Benton and Davis (1977).

4.2.5.1 Oligonucleotide Probes

Hybridizations of the filters using oligonucleotide probes were carried out overnight after a two hour pre-incubation in a buffer consisting of 2 x Denhardt's solution, 6 x SSC, 0.1% (w/v) SDS, 0.1 mg/ml yeast tRNA and 0.05% (w/v) sodium pyrophosphate. In each case the filters were washed twice at room temperature for five minutes after the overnight incubation, followed by two fifteen minute washes at the hybridization temperature. Stringencies of hybridization and washing were varied according to the length and degree of degeneracy of the oligonucleotide probe. Details for specific experiments are provided in the Results section (4.3.1 and 4.3.2).

4.2.5.2 Random Primed Probes

Hybridizations utilizing the random primed genomic fragments were carried out overnight after a two hour pre-incubation in a buffer consisting of 5 x Denhardts' solution, 5 x SSC, 0.1% (w/v) SDS, 200 µg/ml denatured salmon sperm DNA and 1mM EDTA. Hybridization conditions and stringency of filter washes are presented for individual experiments in the Results section (4.3.3-4.3.5). The procedure for washing filters was the same as for oligonucleotide probes (section 4.2.5.1).

Filters were subjected to autoradiography at -80°C (with Du-Pont intensifying screens) on X-ray film (Fuji-RX). Exposure times varied with signal strength.

4.2.6 Isolation of Poly A+ mRNA

Approximately 3µg of poly A+ mRNA was extracted from 150 brain/retrocerebral complexes of pharate and newly eclosed adult *H. armigera* of mixed sexes, using the Pharmacia 'Quickprep mRNA Purification Kit'. This procedure utilizes guanidium thiocyanate to isolate RNA and protect it from degradation, followed by the selection of poly A+ mRNA using oligo (dT)-cellulose. Initially poly A+ mRNA is bound to the oligo (dT)-cellulose under high salt conditions, then impurities are removed with five washes in

a high salt solution (0.5M NaCl in TE; 10mM Tris-HCl (pH 7.4), 1mM EDTA) followed by five washes in a low salt solution (0.1M NaCl in TE) solutions. Poly A+ mRNA is liberated from the oligo-dT cellulose in TE.

4.2.7 cDNA Library Construction and Screening

The synthesis of cDNA from poly A+ mRNA was a two step process. Firstly, a DNA strand complementary to the mRNA was produced using Moloney Murine Leukemia Virus reverse transcriptase. Random hexamers (7.4µg) were used to prime the first strand cDNA synthesis reaction from 3µg of poly A+ mRNA. Secondly, double stranded cDNA was produced by incubating the first strand cDNA with RNase H, which introduces nicks into the RNA strand of the cDNA:mRNA duplex, and DNA polymerase I, which uses the nicks as a priming site to synthesize double stranded cDNA. *Eco RI/Not I* adapters were ligated to the cDNA molecules, and in turn ligated into the *Eco RI* site of the phage vector λgt11. This preparation of cDNA for cloning was done with a 'Timesaver cDNA Synthesis Kit' (Pharmacia) and packaged into phage particles using the 'λ *in vitro* packaging Kit' (Amersham).

The ligation and packaging of 100ng of cDNA into 2µg of λ vector yielded approximately 150 000 pfu, of which 95% were estimated to contain a cDNA insert. The percentage of non-recombinant phage was determined using a blue/white plaque selection system (Sambrook *et al.*, 1989).

The cDNA library (approximately 120 000 pfu) was screened as described for the genomic libraries (section 4.2.5), except that the *E. coli* host strain Y1090 (*lac*-) was used.

4.2.8 Preparation of DNA for Sequencing

Regions of isolated phage clones hybridizing to probes were identified by restriction enzyme digestion (commonly Pharmacia and Promega enzymes) of the phage DNA, followed by agarose gel electrophoresis, Southern blotted onto Nytran (Schleicher and Schuell) (all as described by Maniatis *et al.*, 1982) and hybridized as described in section 4.2.5.

DNA fragments of interest were isolated from agarose gels using either the 'Geneclean kit' (Bio-101) or the 'Sephaglass BandPrep Kit' (Pharmacia). This technology relies on dissolving the agarose gel in a NaI solution and binding the DNA to a silica matrix. Impurities are removed by three high salt solution washes, followed by elution of the DNA with TE.

Fragments of DNA isolated in this way from the *H. armigera* or *M. sexta* genome were ligated into appropriate sites in the plasmid vectors pTZ18u (BioRad), pTZ19u (BioRad), pBluescript SK- (Stratagene) or pBluescript KS- (Stratagene), all of which

contain an ampicillin resistance gene as a selectable marker. Recombinant plasmids were transformed into competent JPA101 cells (tetracycline resistant), using a $CaCl_2$/heat shock method (Maniatis, *et al.*, 1982) and plated on to LB plates containing the appropriate antibiotics.

DNA fragments less than 400bp in length could be completely sequenced without further treatment. For large fragments, a set of unidirectional, overlapping deletions were constructed using the Pharmacia 'Nested Deletion Kit'. This procedure uses blunt or 5' overhanging ends generated adjacent to the DNA insert as a binding site for the controlled, single-stranded digestion of this insert with exonuclease III (Henikoff, 1984). A 3' overhanging end, which is 5' of the first cleavage site ensures that the enzymatic deletion of the DNA is unidirectional.

4.2.9 DNA Sequencing and Sequence Analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using modified T7 DNA polymerase (Pharmacia). Regions of strong cross-banding or compressions were resolved with the 'Deaza G/A Sequencing Kit' (Pharmacia) which contains modified guanine and adenine residues designed to reduce intermolecular hydrogen bonding and so destabilize secondary structures.

Samples were electrophoresed at a constant current of 32 mAmps on a 6% acrylamide denaturing gel (gel dimensions; 42 x 30 x 0.02 cm). DNA was fixed in the gel after electrophoresis by soaking in 10% methanol/10% glacial acetic acid for twenty minutes. The gel was then transferred to 3MM paper (Whatman) and dried in a vacuum gel dryer (BioRad-Model 583) at 80°C for one hour. The dried gel was autoradiographed overnight at room temperature.

4.2.10 Computer Aided Sequence Analysis

Sequences from *M. sexta* which were used to construct the codon usage table (Figure 4.1) were obtained from the GenBank database (release 73) (Bilofsky *et al.*, 1986). Database searches were carried out through the National Center for Biotechnology Information, Bethseda, USA, using the BLAST network service (Altschul *et al.*, 1990). Sequence analysis was undertaken using the GCG package (version 7) developed by Devereux *et al.* (1984). A brief description of those programs specifically referred to in the Results section is given below.

a) 'Bestfit'; gives a best alignment of the optimal segment of similarity between two sequences (Smith and Waterman, 1981).

b) 'Gap'; gives a best alignment of two sequences over the entire length of these sequences (Needleman and Wunsch, 1970).

c) 'Pepplot'; (only 'option j', hydropathy and hydrophilicity was used); predicts the

hydrophobic/hydrophilic regions of a protein sequence (Kyte and Doolittle, 1982).
d) 'Codonpreference'; identifies possible protein coding sequences within nucleotide sequences by analysis of the base composition at the third position of codons, which is statistically more likely to either be a G or C in protein coding sequences (Bibb *et al.*, 1984).

4.3 RESULTS

4.3.1 Screening the *H. armigera* Genomic Library with Oligonucleotide Probes

Two attempts to isolate the Hea-AT gene from the *H. armigera* genomic library were made using oligonucleotide probes designed from the Mas-AT peptide sequence.

In the first screening, the Masat 1 probe identified 10 positive clones (hybridization conditions; 30°C and washes at 30°C in 2 x SSC). Counter-screening of these 10 phages with the Masat 2 probe left one positive. A 1.5kb *Xho I* sub-clone of this positive phage was sequenced and a 15bp region which could code for the first five amino acids of Mas-AT was found. However gene sequences which may code for the eight C-terminal amino acids could not be identified in the remaining 350 downstream nucleotides nor was there any evidence of an exon/intron boundary at the 3' end of the 15bp sequence (data not shown).

In the second set of screenings, Masat 4a/c was the oligonucleotide probe used during the three rounds of screening (hybridization conditions; 45°C and washes at 45°C in 4 x SSC), in which seven positive phage clones were isolated. Each of these phage clones was further characterized by restriction enzyme analysis to identify smaller fragments that hybridized with Masat 4a/c (Figure 4.2). Sequencing and computer analysis of the eight sub-clones failed to identify a *H. armigera* genomic sequence that could encode a peptide similar to Mas-AT (Table 4.1). Translation of the genomic sequences in both orientations and in each of the three reading frames, at best uncovered a region of *H. armigera* DNA that could code for 5 amino acids of the Mas-AT peptide (Table 4.1). For several clones the degree of sequence similarity to Masat 4a/c does not appear to be significantly greater than that expected for a random sequence. This is illustrated by comparison of the Masat 4a/c sequence with that of the plasmid vectors pTZ18u, pTZ19u and pBluescript SK- (Table 4.1), which showed levels of sequence homology similar to that observed for some *H. armigera* genomic sequences, despite the fact that the oligonucleotide probe did not hybridize to the vector (Figure 4.2).

Although all cloned sequences were submitted to a database search through the BLAST network service, none were found to have significant homology to any other

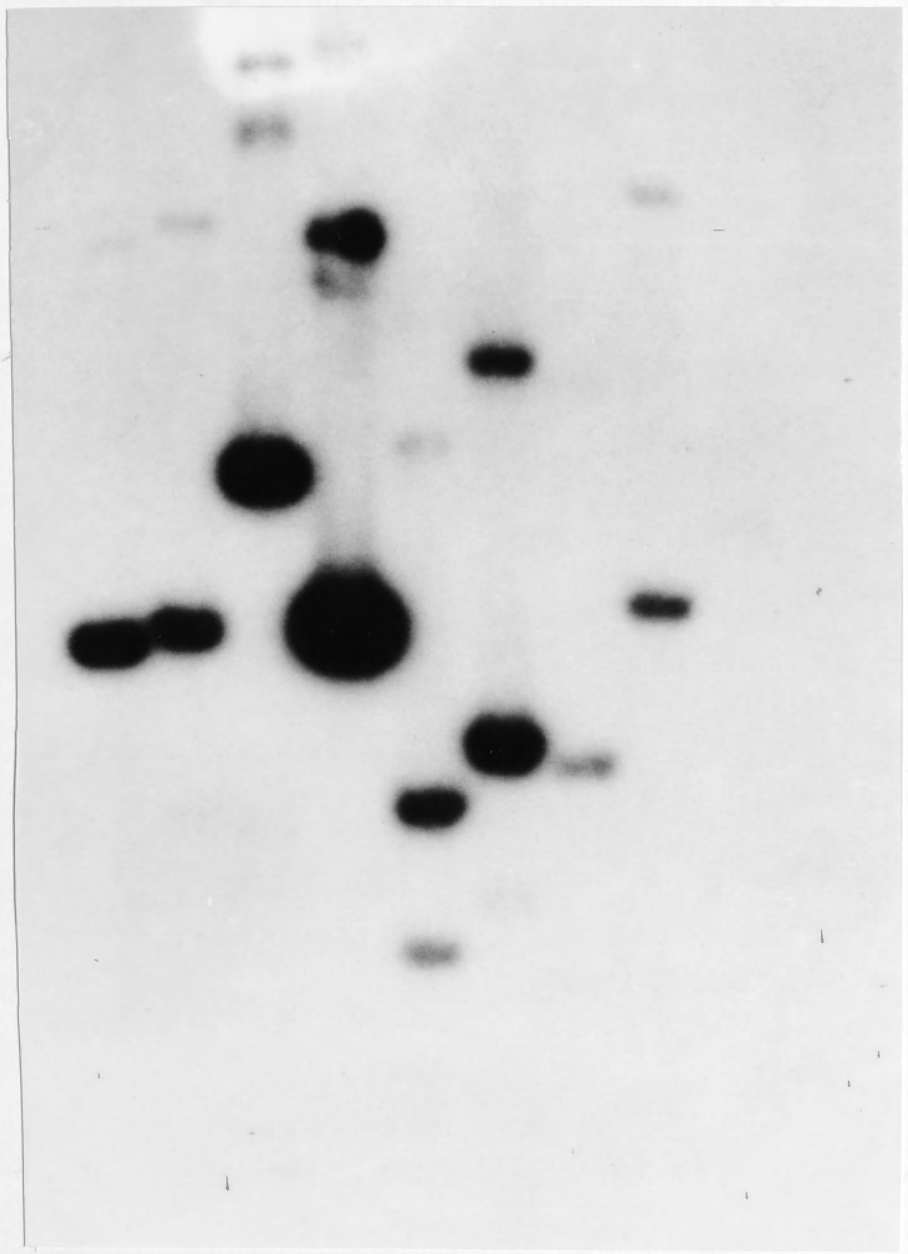


Figure 4.2; Hybridization of Mas-AT 4a/c to sub-clones isolated from the screening of the *H. armigera* genomic library using the oligonucleotide as a probe (hybridization conditions; 45°C, washes at 45°C in 4 x SSC). Phage clone 23 contained two hybridizing regions, designated as 23 and 23T1. Sub-clones 13 and 15 presented in this figure were sub-cloned further before sequencing. Multiple bands are detected in most of the lanes because the DNA was not cut with restriction enzymes and so multiple forms of the circular DNA molecules can be seen (e.g., open nicked circles, supercoiled, etc). In Lane 1 is the sub-clone 1B8, Lane 2, 5HB3; Lane 3, 13; Lane 4, 14H1; Lane 5, 15; Lane 6, 22; Lane 7, 23; Lane 8, 23T1; Lane 9, the plasmid vector pTZ19u and Lane 10, the plasmid vector pBluescript SK-.

SUB-CLONE		BASE HOMOLOGY		AMINO ACID HOMOLOGY	
Name	Size(bp)	(%)	Length(bp)	(%)	Length(aa)
1B8	1578	82	17	40	10
		91	23		
5HB3	1736	71	21	100	4
		76	25		
13	1976	77	26	100	4
		78	18		
14H1	1681	78	18	100	5
		100	15		
15	202	71	24	43	14
		82	22		
22	728	100	10	100	5
		89	19		
23	651	93	14	57	7
		74	19		
23T1	1985	87	15	36	11
		73	26		
pTZ	2860	84	19	80	5
		88	17		
pBL	2961	74	23	80	5
		88	17		

Table 4.1; The highest nucleotide (both orientations) and translated protein similarities of the eight *H. armigera* sub-clones, hybridizing to Masat 4a/c, to all possible coding sequences for Mas-AT and Mas-AT peptide sequence respectively. Sequences of the plasmid vectors used in the sub-cloning experiments were also included in the analysis. Data was obtained using the Bestfit program (Smith and Waterman, 1981). pTZ represents the plasmids pTZ18u and pTZ19u, whereas pBL represents the plasmid pBluescript SK-.

sequences which have been reported.

4.3.2 Screening the *M. sexta* Genomic Library with Oligonucleotide Probes

After two rounds of screening (hybridization conditions; 50°C and washes at 50°C in 2 x SSC) with Masat 6, eleven potential positives were detected. A tertiary screening with Masat 4a/c II and quaternary with Masat 7 (Masat 7 hybridization conditions; 60°C and washes at 60°C in 2 x SSC) were carried out in an attempt to reduce the number of false positives. After the tertiary screening three positive clones remained, all of which re-screened with Masat 7. DNA of these three phage clones was digested with various restriction enzymes, Southern blotted and hybridized with Masat 6 (hybridization conditions; 50°C and washes at 50°C in 2 x SSC). A 2.1kb *Cla I* fragment from each phage clone hybridized with Masat 6. The *Cla I* fragment was sub-cloned from each phage and preliminary sequence data (approximately 400bp in each case) showed that the same region of the *M. sexta* genome had been sub-cloned.

One of the *Cla I* sub-clones was selected and sequenced in its entirety. A region of DNA was found within the sub-clone which would code for Mas-AT, including a C-terminal glycine (Figure 4.3). A 2.3kb *Sal I* sub-clone (from the same phage as the *Cla I* sub-clone), overlapping the *Cla I* fragment at the 3' end, was also sequenced to complete the sequence of the open reading frame which putatively encodes the Mas-AT peptide (Figure 4.3).

4.3.3 Isolation of the Putative Hea-AT Gene Using the Putative Mas-AT Gene Sequence

The *H. armigera* genomic library was screened with a 430bp *Acc I/Eco RV* fragment of the Mas-AT genomic phage clone (see Figure 4.3) which spanned the complete putative open reading frame of the Mas-AT peptide (hybridization conditions; 50°C and washes at 50°C in 2 x SSC). After the first two rounds of screening, four positive clones were isolated. These clones were counter-screened with Masat 4a/c II, and one positive clone was identified. Restriction enzyme cleavage, Southern blotting and hybridization analysis identified a 2.6kb *Hind III/Sac I* fragment which hybridized to both the Mas-AT *Acc I/Eco RV* fragment and Masat 4a/c II.

The 2.6kb *Hind III/Sac I* fragment was sub-cloned, sequenced and found to contain a region which could code for a peptide identical to the Mas-AT, including a C-terminal glycine (Figure 4.4).

A 'Bestfit' alignment of the Hea-AT and Mas-AT open reading frames identified a 113bp region which is 75% identical between the two nucleotide sequences (Figure 4.5) which extends over the regions putatively encoding the Hea-AT/Mas-AT peptide.

```
      Cla I
   1 ATCGATAGAATTTCTTCGAAACTAGATGCATCTTACATATTATAAAACATAGTCCTTAGTCGAGTCCGTCTGAATGCGAT
  81 GACTCAATCATTACGGAATGGGTTTCGTTGAGATTTTGTACGGTGACAGTTTGAGGCCCAGGGAAGGACATAGGCTACTT
 161 TTTATTCCGAAATTAAATATGTAGCAAGACTAATACAGGAAAAGTATACACGCGGGCGAAGTCGCGAGAAAGGTTAACAA
 241 ATGTTACCAGGAAAAAAAAACTCTTACCATACCTATTGTGGTGAAAATTAGAAGTAATTCCTGGTACTGGCAATTACAAC
 321 TTAAAAATGAAAAACCAATACGGCGCGCCCAAAGCATTTTCACACAATTTTTTGACATAGCAAGTCAGGCTTGAAGTCAA
 401 TAATTTGTGTTTTCGAAAAAATTACATAAAAAACAATTATATTGCAAAAATATATGTTGGTTAACTTATTCTTACAGACT
 481 AATGCTGTAAATAATATTCCAGTTATTGTACTAAGATAATGCTGTTAAAACTAGGAAGGTAGAACAAATGATAACCTCAA
                                  Sal I
 561 AGTGTCACTATTCATAAAACTACAATGGCATTGTCGACGCTTTGGCCACTCCGCAAGAATCCGATAACTAAATTATTGGA
 641 CACACTGCTTCTCTCGTATTATTTAATGGAAAAGTCCATGTTTTCACATTAGAACAAATTGTGTTATTTCCAAGAAAAGC
 721 TGTTTGAGAGCCCATTTTTCATTTATCAGATAAAAAATATCCGTGGTATACATTATAACATGACTAAATATAAAAGTAAT
 801 CAAATTAAGTGTTTTATTAATGTAATTCTTATTGTTAATTTATTTGTGTTATCCTATGAATAAAATTAATTTGAAAAAAA
 881 CAGGCTTGATATTGAAGATTCGTTTTGCGCAATTTCAGAACTTATTGCTTTCTCTAATAACTTCATTGCAAGTATGTTAA
 961 CTACAACCACTGAAGTTTCTTCTCTTTAATAATCTTGAAAGTTGAACGCCTCTCGGTATAGATATATTTCAAAACTTTTT
1041 AATTTAGTTATGTATTGCTCTATTTTAATATCTGAGATGTTGAAGGAGATATTATGAAGATATTACAATAATAATTTTGT
1121 GAATATTCCCACCATTTTCTTTCCATTTTCCAATTGACTGAGCATATTAATTATATATTTTTTTACGTAAATCACTTTGC
1201 GATGTAAGTAAATTTATACTAAGTAATTTTAGAGGAAAATTATCACAAGTATGTGTTAGCAGTTTCATGAGAAATTTAAA
1281 ATATTACAATTGTTTCTTCAATCAAGATACTCCACATATAATGATTTCTTATGTTTTTCGCGAATTTTAGACATTAAAAT
1361 TAAACCTCCCTCCCGTGACCACGTAGGCTGCAAAGTCTTCGAAAGGTCGGGAGAAAATTAAAATATAAAAACCGCGATAA
1441 TATCCGAAACATAGTTTAATTTTAATGCTCAATAAATATTATCTAATAGCGGCGATAGCCTAGTTGGGTGTGGAACGGAC
1521 TGCCAAGACGAATGTCCGCAGGTTCAAATCCCAAGGGCACACACCTCTGACTTTTCTTAAATCATGTGTGTATTCCTTGT
1601 GAATTTATCGTTCGCTTTAACGGTGAAGGAAAACATCGTGAGGAAACCTGCACATGTGAGAAGTTCTCTATAGGAATTTC
                  Acc I
1681 GAAGGTGTGGGAAGTCTACCAATTCGCACTAGGCCAGCGTGGTGGACTAAGGCCTAATCCCTTTCAGTAGTAGAGGAGGC
                                                                    ArgArgP

1761 CCGTGCTCAGCAGTGGGCAAGTGTATAATACAGGGCTGATATTATTATTATTATATAAATATTATTTTGTTTCAGAAATG
     roValLeuSerSerGlyGlnValTyrAsnThrGlyLeuIleLeuLeuLeuTyrLysTyrTyrPheValSerGluMet

1841 AATCTGACAATGCAACTGGCGGTGATCGTGGCTGTGTGCCTCTGCTTGGCGGAGGCGCGCGCCGACGTGCGGCTCACGAG
     AsnLeuThrMetGlnLeuAlaValIleValAlaValCysLeuCysLeuAlaGluAlaArgAlaAspValArgLeuThrAr

1921 GACCAAGCAACAGCGACCCACGCGCGGCTTTAAGAACGTCGAGATGATGACTGCCAGGGGCTTCGGAAAGCGCGACAGGC
     gThrLysGlnGlnArgProThrArgGlyPheLysAsnValGluMetMetThrAlaArgGlyPheGlyLysArgAspArgP

                                                          Cla I
2001 CCCACCCCCGTGCCGAACGTGAGTTTAAGTTTATACTTTGTTCTAAAATTTTCCAGAATTATCGATATTATTATAACTGA
     roHisProArgAlaGluArgGluPheLysPheIleLeuCysSerLysIlePheGlnAsnTyrArgTyrTyrTyrAsnEnd

                                                          Eco RV
2081 CTGATAAAATCAATCAACGTTAATTACATACTGTTAATTGTTATAAGCGTAGATTATCGATATCTATATAAACTATTTGT
2161 AAATAAGGTCTTGATAATTTTGCGATAGGTCCCACAAATTATTTGCTTCTATTAAGTCCCTAATCCCCCAATGTAAAGGT
2241 CGGCAACGTATTCGTTTTTCTGCCAGCATAAGTGGGCGTAATCCAGCGTGGTTATACGTGACTTATCGGCTCATTTACCT
2321 TCCTTTATAATGAATACATCGCTGATTCAATATTATAAAGTTTTGGGTTAATTTTTATTTATCTATGAAAAATGAGATTT
2401 AATTTTTCATGTTAGTAAAAATGTAATAAATAAGTTAATGGCTCTCTCTTGGAATATCTTACGGGGTTACGGATTTATTT
2481 TTTACTGGAAAAAACATTTATCATGTGATACATAATTTAATTGAACCTTGTAAAAGTATGCCAGCGTAATGTATGTTTTA
2561 ATAAAAGATATGTTTCAATTCCTGTATAGTTTCATGTAACGTATAATCTTTATACTTAATATTTTCTACTGATTTTGTTA
2641 CATAGACGCGTTTTATGAGCTAGACGAATGTTTTATTTCAGACCACGCGTGTCGTGTGGCATTAACTTGCGAAAACATAT
2721 TTGATTTATTGAAATAATTCTATGAATTCAATGTCAGTTTAAAATTTATTGTGACTAACACATGATATCATAAAATGTTT
2801 GTAGATACAGAAATGGATATTATAAAGATTAGAATTAAAGAGACAATAACATTTTGAAATATATAAATTGTCATAGTATA
2881 TAATACATTAGTATAGATAACAAAGTGATCACTTCAAATAAAATACAACGACAATTACGGTAAATTTTGaATATTCTACC
                  Sal I
2961 GTACAAAAAAAATGTCGAC
```

Figure 4.3; Complete nucleotide sequence of the 3kb *Cla I* to *Sal I* region isolated from a *M. sexta* genomic library using oligonucleotides designed from the Mas-AT peptide sequence. The translation of an open reading frame which would encode the Mas-AT peptide is shown. The Mas-AT peptide is underlined and italicized. The region between the *Acc I* and *Eco RV* sites indicated was used as a probe to isolate the homologous region from a *H. armigera* genomic library (section 4.3.3).

```
    Hind III
   1 AAGCTTATTACTTCACATATTACTTGTTTGTTGTTTCAGATAGCACTTTAATTAAACTATAAGCCCCAGCTGCTCATGTT
  81 ACCCCTTTAAAAAATCAAATAGCAATTTCATACATTTAGCCATTGAATTTCGATGATGCCTCCTATGCAGTATTTCATTA
 161 CTTATTATTACCTATAATTTGAGTTACTTTTCGTTTGCGCCGGGCTTGGGTTTTTTTTGAGCATTGTCCTTTGTTTTAGA
 241 TGAAATTAGATTTTTTTTATGAGAGATTTAGGTGTCGTTTTATTTGACTAAAATATTGTTGTTTTGAGTACTTAAATAAA
 321 AATTAAAAAAAGATTATAGCTAAGACCTCTTTCATCGACTGTCAGAGAAGGCACATTCGAAATCAAGATATAGTTTTTTTA
 401 CACTACTGTCACGCTATCACATACAATTCCATCTTATCGAAAGGCACTTAATAGCCTACGCTCTTTAAGAATACATTTCG
 481 GAAATAATACAAACATTTGGGGCTATTTTCACTAACAGCATAAAAGTCAGTAGGTATTCTTGAAACAACTGATAGGTAAC
                                                                          ValTh

                                                          Dra I
 561 TGATTTTCAACGTCATTCTCAAAGTAAACAGTTGTTTTCAGAAAAAAACGGGCGTTTAAATTCTCTTAGCATGGTTCCAA
     rAspPheGlnArgHisSerGlnSerLysGlnLeuPheSerGluLysAsnGlyArgLeuAsnSerLeuSerMetValProI

 641 TTACTAACGTTTCAATATCTTTGTCCCCAGCAATGAATTTCTCAATGCACCTGGCAGTAGCAGTTGCAGCAGCGGCCTGC
     leThrAsnValSerIleSerLeuSerProAlaMetAsnPheSerMetHisLeuAlaValAlaValAlaAlaAlaAlaCys

 721 CTCTGCATAGTGGCCGCAGCCCCCGAGGGGCGGATCACCCGCACCAAACAGCAGCGCCCCACCCGTGGCTTCAAGAACGT
     LeuCysIleValAlaAlaAlaProGluGlyArgIleThrArgThrLysGlnGlnArgProThrArgGlyPheLysAsnVa

 801 CGAGATGATGACCGCCAGGGGCTTCGGCAAGCGCGACAGACCACACACTAGGGCTGAGCGTGAGTTTGAAGTTTGAATTT
     lGluMetMetThrAlaArgGlyPheGlyLysArgAspArgProHisThrArgAlaGluArgGluPheGluValEnd

 881 GGAACTTTATTGTTTGTTTTTCTCTTTTATTTTAATAAATAAAAAAAAATCTCTTTTATTTTACTTATCTTGTAAAGGAA
                                                          Cla I
 961 AGTGTTTGAATTTAGAATGAGTTTATTCTTTTTAATCATTTCGGTATCTACCACGATAAATCGATGTTCTGCACAGATAA
1041 AAAGTACTCTTTTTTTTTAGTAAGTCAAAATTTTTGCCACATTAATTAACAAAAATAATGTTTCTGTGTAAAAATCACAT
1121 TTGATGTGTAAACATGCACTGTATGTATGTAAGTAGACGTTTAACTTTTAAATATAATATCAGGTTTTCTAGGGGTGCAT
1201 AAGTGGTTTATTTAAACATGTACCTATACATCGATCGTACCTCTAGTTGTTTTGTATATTATGTGTTTGTATAATTCAAC
1281 TTATTTTCAGATTTACAGTTAAAGTGTATGATAAGGAAATTAAACTTATTCCCTAATAAACATAGGTAAAAATATGTTCC
1361 TAAGTTTTAATATTCTATTACGGTAAAACGTAAATACAAGTGGAAATATGAAAGATTAATTAAGTTTGCTAGAAACGACA
1441 GCCGTTATGTTTAAGTACGTAATGGAAATTGGGAATGTGTATAATTAACACTAATTGTATGGCATTAATAAACCACCATA
1521 CATCATGTTAACTCAACACTAACTCTTCAGAAACTTTTGCTTTTATGTTTACTAACATGCTGAAAAAACTAACTACACGG
1601 AATATTTTAATATCATTACTTTGCCTCAAACTTCCACCGAGTTAGGATATCAATCAGAAATTGTTGTATAGTAAAAAGAT
1681 TATGCGGCATTTTATGAAGTAATTAGGAACATTTGTACAGGGTGATGTATGTGAGGAAGTTCACAATAGTGAGGCATCCA
1761 TCTTCCTAGCCTGCCAACTTATAAGTTGCGCTACTGATCGCTAATGGAGCGAATAATTCGTACAGAGTAATATATCGGGA
1841 ACACATTCCTCCAACCTTTATATTAGCCTTTATTGAAATTCAAACAACCTCAATTGAACGGAAACTCGTGCAACTTTTCA
1921 ACCCTCATTGTTCGATATGTAAAGTTCGAAATATTCTATCGAAATATTTCGTATTACCATAATCTTTATGAAATTGTGCT
2001 TTCAAAAGCACAATACAATATTGTTAATATTTGTTATCGAAGGGTAAATAAGTTTACACTGGGTTCCCGTGTCTCGGTGG
2081 GACGGTTTGAAGTGACTGTAATGAGGCTAAGAATGGTAACATTATTCCTGAAGGGGACGTTGAGCACCAAGCGCCGAACG
2161 AGCCGCTCGCACCGCGGCACGCCCACCTTTAAGAGTCCCACAGTTGGGATCGCGAGGGATTTCGGTAAAAGAGCCCCGGA
2241 ATTGGACGGTGAAGGTACCACAAAGAGTCTGATTGGATTGCCGGCCTCGCGTCTCTGTGAATGGCAAAAATTACGTGGAA
2321 GCCGACGAATGCCGGACCGTGTAACAGACTTAAACACAGTCCAAACATGCGATACTTTGGAACGAGCCCTTCGGAAGCTC
2401 CTCGCTTTGCAGATGTTTGTGAGAGGATACAAACCTCGTTACCGCTCGATATTACGTAGGTTCACTACGAAGAACCGTCT
2481 CCGTAACTCCTTTACTCAGAGCGAAGTCACGAATAGTATTCCGCATCGCATCGAACGCAAATAAACTGTATTTTCAGTCA
          Sac I
2561 ATAACAGAGCTC
```

Figure 4.4; Complete nucleotide sequence of the 2.6kb *Hind III/Sac I* clone isolated from a *H. armigera* genomic library using a probe from the homologous region of the *M. sexta* genome (Figure 4.3). The translation of an open reading frame which would code for a peptide exactly the same as Mas-AT is shown. The putative Hea-AT peptide is underlined and italicized. The region between the *Dra I* and *Cla I* sites indicated was used to isolate the Hea-AT cDNA (section 4.3.4) and map the Hea-AT gene structure (section 4.3.6).

```
 Length:                214 bp    Gaps:                  1
 Percent Similarity: 75.481    Percent Identity: 75.481


M. sexta
    1833 CAGAAATGAATCTGACAATGCAACTGGCGGTGATCGT......GGCTGTG 1876
         ||| ||||||| |  ||||||| ||||| ||    ||        || |
     668 CAGCAATGAATTTCTCAATGCACCTGGCAGTAGCAGTTGCAGCAGCGGCC 717
H. armigera

    1877 TGCCTCTGCTTGGCGGAGGCGCGCGCCGACGTGCGGCTCACGAGGACCAA 1926
         ||||||||| | | ||  ||   | |||| | |||| |||| | |||||
     718 TGCCTCTGCATAGTGGCCGCAGCCCCCGAGGGGCGGATCACCCGCACCAA 767

    1927 GCAACAGCGACCCACGCGCGGCTTTAAGAACGTCGAGATGATGACTGCCA 1976
          || ||||| ||||| || ||||| |||||||||||||||||||| ||||
     768 ACAGCAGCGCCCCACCCGTGGCTTCAAGAACGTCGAGATGATGACCGCCA 817

    1977 GGGGCTTCGGAAAGCGCGACAGGCCCCACCCCCGTGCCGAACGTGAGTTT 2026
         |||||||||| |||||||||| || ||| |  | || || |||||||||
     818 GGGGCTTCGGCAAGCGCGACAGACCACACACTAGGGCTGAGCGTGAGTTT 867

    2027 AAGTTTATACTTTG 2040
           |  ||  | ||||
     868 GAAGTTTGAATTTG 881
```

Figure 4.5; 'Bestfit' alignment of the Mas-AT and Hea-AT genomic clones (from Figures 4.3 and 4.4, respectively). The sequence coding for the Mas-AT and Hea-AT peptide is underlined.

Database searches with the nucleotide sequences found, at best, gene sequences of 65% similarity over 43 nucleotides.

4.3.4 Isolation of a cDNA Clone of the Hea-AT Gene

The adult *H. armigera* brain cDNA library was screened using a 410bp *Dra I/Cla I* fragment from the Hea-AT gene sequence, which spanned the complete region putatively encoding the Hea-AT preprohormone. After three rounds of screening at 60°C and filter washes at 0.5 x SSC, two positive clones were isolated.

Cleavage of these clones with *Eco RI*, which liberates the cDNA insert, found one positive phage had a 1.6kb insert, and the other a 4.2kb insert. The clone with the smaller insert (hereafter referred to as the Hea-AT cDNA clone) was characterized further as it consistently hybridized more strongly to the probe.

The Hea-AT cDNA clone was sequenced (Figure 4.6) and when compared to the Hea-AT *Hind III/Sac I* genomic clone (Figure 4.4) was found to contain a 168bp region which is identical between the two sequences and encompasses the entire sequence coding the Hea-AT peptide (Figure 4.7). However both 5' and 3' of this region the sequences are unrelated. Therefore it appears that the Hea-AT gene consists of at least three exons; the Hea-AT coding exon, and at least one exon both 5' and 3' of the Hea-AT coding exon.

The points at which homology between the Hea-AT genomic DNA sequence and the cDNA sequence cease should indicate intron-exon splice sites. Accordingly, the Hea-AT genomic DNA sequence at these regions was examined for similarity to consensus acceptor and donor splice sites (Shapiro and Senapathy, 1987). Hea-AT genomic sequence at the 5' junction (Figure 4.7) conforms to the AG-rule (Shapiro and Senapathy, 1987) and flanking sequence is in good agreement with that described for the splice acceptor consensus sequence (Figure 4.8). In addition, homology between the Hea-AT and Mas-AT genomic sequences begins in this same region (Figure 4.5), a result consistent with presence of an intron-exon boundary at this position.

In contrast, examination of genomic sequence at the 3' end of the Hea-AT genomic DNA/cDNA alignment (Figure 4.7) failed to identify sequence with significant similarity to the donor splice site consensus sequence (Figure 4.9). Comparison of the Hea-AT and Mas-AT genomic sequences indicates that significant homology continues for a further 46 nucleotides. The possible significance of these results is considered in the discussion (section 4.4.2).

4.3.5 Hea-AT Gene Structure

A 290bp *Eco RV* fragment, spanning the most 5' region of the cDNA clone (shown in Figure 4.6) and a small portion of the vector multiple cloning site, a 180bp

```
   1 AATTCGCGGCCGCTCGTGTAATATGAAATTGAAAGTGTCCGCACCGTGACGCGGGTTCCCGCTAGAGACTGTGCAAAAAT
  81 ATATAACTGGTGCAGGCGCGGGCGATCCCCACGATACATCTTTACAACAAACGTTATGTATACATATAGCTATAGTAATA
 161 AGAATATCTATTTGTTATATTATCAACAAAATATTAGATCAGAAGTGAGGGTGCGTCCGCCCCGCTCTGCCTCCCGCCTC
                                                    Eco RV
 241 TACTACATCACACCATGATATGACATCGCGATCTATCATATACTTAAACACAGATATCTCAATATGATACACTCTGACTT
 321 AACTAGGAGATAGCAACAGCGCGCGTAATATTTAGCCTTAGATGGAGTTTACCACCCACTTAGGGCTGCACTCTCAAGCA
 401 ACCCGACTCTAAGGAGAGTCCCTCTCGCCACGGTCCTCCGTCGCTACGGGCCTGGCACCCTCTGCGGGAAAACGGCCCCG
 481 TTCAAGACGAACTTGGACAGGAGCGACCGCGACGAGAAAGCGGAACCTCCCGAACACCACATCTCCCGCCGCCTGAACAA
 561 CGGCGGGATTCAGTGCTGGGCTTCATTCCTGTTCGCTCGCCGCTACTAAGGAAATCCTGGTTAGTTTCTTTTCCTCCGCT
 641 TACTAATATGCTTAAATTCGGCTTTAATCTATGACTTTATTTTTAAATATCTACTGGTACCACAGTGGGGTCGTAAACCC
 721 CATTGGTTTCCACCTTGCCTTCGTACACGATCTGCTGAAGCTGGTTCTCGTAGCCATGAGGTCCTGATTCTTCCATCTTC
 801 TTCAGCAAACTCCTTATTAAGATTATGAGGTAGATTTGGATGGCTAACGCAGCGAACATGAGAGGCAGTACTTCGAAGAC
 881 CATGTAGAAACCCATCCTACTGGACACAGAGACCGCGAAGTCTAATATCTGGATGAGCACAGACGCTACCGTCGTGGTCA
 961 CGGCGAAGTAGTAGTACTGTTTTTATGTACAGGATTAGTTTCTTATGAGCTCCATACAGCAGCACGGCAGAGAACACAAC
1041 TTCTACGCAGTAGATGAAGACACAAACTTCTTCAGGTATTGACACACCAACACTGGTCCCATGGTAGATCGCAAATAAAC
1121 CGAAGTCTGCGTGAACGGAGTAGCTGTAGATCCCCACCATGAACGCGCTGAAAATTAGGCAACACGCGCTCTCAGCAACT

1201 GTGTTACAGTGAACTACTCGTACAGTTACGGACAAGACAAACTTGCAGCAATGAATTTCTCAATGCACCTGGCAGTAGCA
                                                    MetAsnPheSerMetHisLeuAlaValAla

1281 GTTGCAGCAGCGGCCTGCCTCTGCATAGTGGCCGCAGCCCCCGAGGGGCGGATCACCCGCACCAAACAGCAGCGCCCCAC
     ValAlaAlaAlaAlaCysLeuCysIleValAlaAlaAlaProGluGlyArgIleThrArgThrLysGlnGlnArgProTh

1361 CCGTGGCTTCAAGAACGTCGAGATGATGACCGCCAGGGGCTTCGGCAAGCGCGTTCGGAATAAGGATTGGCTCTGAGGAC
     rArgGlyPheLysAsnValGluMetMetThrAlaArgGlyPheGlyLysArgValArgAsnLysAspTrpLeuEnd

                                        Eag I
1441 CGGGGCGTGTCGGGTTTGGACGGGAAGCGGATGCGGCCGGTGCCGGGCCTGGTCGATGCTCTGGCGTCTCTCGGGGCGCT
1521 GGGGCGGAATCCGGACCCGCGTTCCGGCCTTCCGCGGATCTTCCTAGCCGTAAGGCCGTGTCGGTTTCGTCTCGTGCGCG
                                                    Eag I
1601 ATCGGCGCGGTTCTGTACGACCGCCGTTCAACGGTCAGCTCAGAACTGGCTCGGAAGCGGCCGCGAATT
```

Figure 4.6; Complete nucleotide sequence of the Hea-AT cDNA clone. The translation of an open reading frame encoding a putative Hea-AT preprohormone is shown. The *Eco RV* (other site within vector) and *Eag I* (other site within the *Eco RI/Not I* adapter) sites utilized to make region specific probes are indicated (see section 4.3.6). The sequences in bold at either end of the Hea-AT cDNA sequence are those of the *Eco RI/Not I* adapters (Eag I site of the 5' adapter is not shown).

```
 Length:                  162 bp  Gaps:                    0
 Percent Similarity:      100     Percent Identity:      100



H. armigera genomic
     650 TTTCAATATCTTTGTCCCCAGCAATGAATTTCTCAATGCACCTGGCAGTA 699
                              ||||||||||||||||||||||||||||||||
    1242                      CAGCAATGAATTTCTCAATGCACCTGGCAGTA 1277
H. armigera cDNA

     700 GCAGTTGCAGCAGCGGCCTGCCTCTGCATAGTGGCCGCAGCCCCCGAGGG 749
         ||||||||||||||||||||||||||||||||||||||||||||||||||||
    1278 GCAGTTGCAGCAGCGGCCTGCCTCTGCATAGTGGCCGCAGCCCCCGAGGG 1327

     750 GCGGATCACCCGCACCAAACAGCAGCGCCCCACCCGTGGCTTCAAGAACG 799
         ||||||||||||||||||||||||||||||||||||||||||||||||||||
    1328 GCGGATCACCCGCACCAAACAGCAGCGCCCCACCCGTGGCTTCAAGAACG 1377

     800 TCGAGATGATGACCGCCAGGGGCTTCGGCAAGCGCGACAGACCACACACT 849
         ||||||||||||||||||||||||||||||||||
    1378 TCGAGATGATGACCGCCAGGGGCTTCGGCAAGCGCG               1413
```

Figure 4.7; Alignment of the Hea-AT coding exon of the Hea-AT genomic and cDNA clones, encompassing the region coding for the Hea-AT peptide and putative exon/intron boundaries of the genomic sequence. The 5' sequence underlined was used in the analysis of acceptor splice sites (Figure 4.8). The 3' sequence underlined was used in the analysis for donor splice sites (Figure 4.9).

A)

	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	INTRON | -2	-1	EXON +1
%A	11 25	12 25	9 24	8 19	11 16	11 26	10 24	10 22	8 12	8 8	25 16	4 1	**100**	0	14 38
%C	30 14	32 21	27 13	29 21	30 18	32 41	38 17	38 33	38 25	35 20	27 16	72 76	0	0	14 9
%G	13 19	12 12	11 13	11 19	10 11	11 7	9 12	8 3	7 0	7 3	24 26	1 0	0	**100**	50 41
%T	46 42	44 42	53 49	51 41	49 55	46 26	44 47	44 42	47 63	49 70	24 42	23 22	0	0	9 12
Invertebrate Splice Acceptor Consensus	T	T	T	T	T	T	T	T	T	T	N	C	**A**	**G**	G
H. armigera position; 657-671	A	T	C	T	T	T	G	T	C	C	C	C	**A**	**G**	C
M. sexta position; 1822-1836	T	T	A	T	T	T	T	G	T	T	T	C	**A**	**G**	A

B)

5' EXON | Hea-AT CODING EXON

Hea-AT cDNA; 1248 - A A A C T T G C C A G C A A T G - 1263

Figure 4.8; A) Nucleotide frequencies at acceptor splice sites. Figures on the left are derived from 1432 eukaryotic sequences, the figures on the right are derived from 86 invertebrate sequences (Shapiro and Senapathy, 1987). The consensus invertebrate acceptor splice site is shown, with alignments of the putative Hea-AT gene acceptor site (shown in Figure 4.7) and the homologous region from the Mas-AT gene. B) The 5' exon/Hea-AT coding exon boundary as determined by the alignment of the Hea-AT genomic and cDNA clones (Figure 4.7). The three nucleotides putatively code for the start methionine of the Hea-AT preprohormone.

A)

	EXON | INTRON								
	-3	-2	-1	+1	+2	+3	+4	+5	+6
%A	36 38	56 63	11 8	0	0	60 75	69 76	7 7	14 17
%C	36 28	13 13	3 5	0	0	3 0	9 11	6 4	14 13
%G	18 25	14 10	78 80	**100**	0	35 21	12 7	82 82	19 4
%T	11 10	17 15	8 8	0	**100**	3 4	11 6	5 7	53 66
Invertebrate Splice Donor Consensus	N	A	G	**G**	**T**	A	A	G	T
H. armigera position; 1992-2000	G	C	G	**A**	**C**	A	G	G	C
M. sexta position; 833-841	G	C	G	**A**	**C**	A	G	A	C

B)

Hea-AT CODING EXON | 3' EXON(?)

Hea-AT cDNA; 1309 - G C G T T C G G A A T - 1319

Figure 4.9; Nucleotide frequencies at donor splice sites. Figures on the left are derived from 1446 eukaryotic sequences; the figures on the right are derived from 80 invertebrate sequences (Shapiro and Senapathy, 1987). The consensus invertebrate 5' donor splice site is shown, with alignments of the putative Hea-AT gene donor site (shown in Figure 4.7) and the homologous region from the Mas-AT gene. B) The Hea-AT coding exon/ putative 3' exon boundary as determined by alignment of the Hea-AT genomic and cDNA clones (Figure 4.7).

Eag I fragment (shown in Figure 4.6), spanning the most 3' end of the cDNA clone, and the *Dra I/Cla I* clone previously described (section 4.3.4) were used as hybridization probes in an attempt to map the exon/intron structure of the Hea-AT gene. Only the *Dra I/Cla I* fragment hybridized to the original *H. armigera* genomic recombinant phage (section 4.3.3) at a hybridization temperature of 50°C and washes at 50°C in 1 x SSC. Examination of a restriction enzyme map of the *H. armigera* genomic phage clone (Figure 4.10) indicates that the most 5' exon is at least 1.7kb upstream of the Hea-AT coding exon, and the putative 3' exon is at least 11kb downstream from the Hea-AT coding exon.

4.3.6 Hea-AT and Mas-AT Preprohormone Structure

The Hea-AT cDNA sequence was analysed using the 'Codonpreference' program (Bibb *et al.*, 1984) which detects both open reading frames and sequences likely to code for proteins (see section 4.2.10) (Figure 4.11). The largest complete open reading frame (frame 3, Figure 4.11) scores particularly well when the third base position of the each codon is examined for GC bias (Figure 4.11). Translation of this open reading frame is presented in Figure 4.6 and includes the putative Hea-AT peptide.

As there is considerable doubt about the putative donor splice site at the end of this open reading frame (see Figure 4.9 and section 4.4.2 for discussion) analysis of the Hea-AT cDNA open reading frame will be confined to a region commencing with the first in-frame methionine and extending to the arginine residue which is three amino acids beyond the C-terminal end of the Hea-AT peptide (Figure 4.6). This 162bp region of the Hea-AT cDNA open reading frame is completely identical to the Hea-AT genomic sequence, but the homology between the two sequences stops beyond the codon for the arginine residue (Figure 4.7). This putative protein was examined for sequence motifs expected of a neuropeptide preprohormone. An hydropathy analysis using the 'Pepplot' program (Kyte and Doolittle, 1982) of the GCG package is presented in Figure 4.12A. The first 25 amino acids of the partial putative Hea-AT preprohormone are hydrophobic in nature and may constitute a signal peptide sequence for the preprohormone (see section 1.4.1). The partial putative Hea-AT preprohormone contains a glycine α-amidation signal immediately C-terminal to the allatotropin peptide sequence (Figure 4.13). C-terminal to this glycine is a dibasic lysine-arginine and immediately N-terminal to the allatotropin peptide sequence is a single arginine residue (Figure 4.13), these basic amino acids are likely to act as endopeptidase cleavage sites to liberate the glycine extended allatotropin from the prohormone.

From the nucleotide sequence comparison of the Hea-AT and Mas-AT genomic clones (Figure 4.5), combined with the translation of the Hea-AT cDNA open reading frame, it can be hypothesized that the Mas-AT preprohormone extends from the first

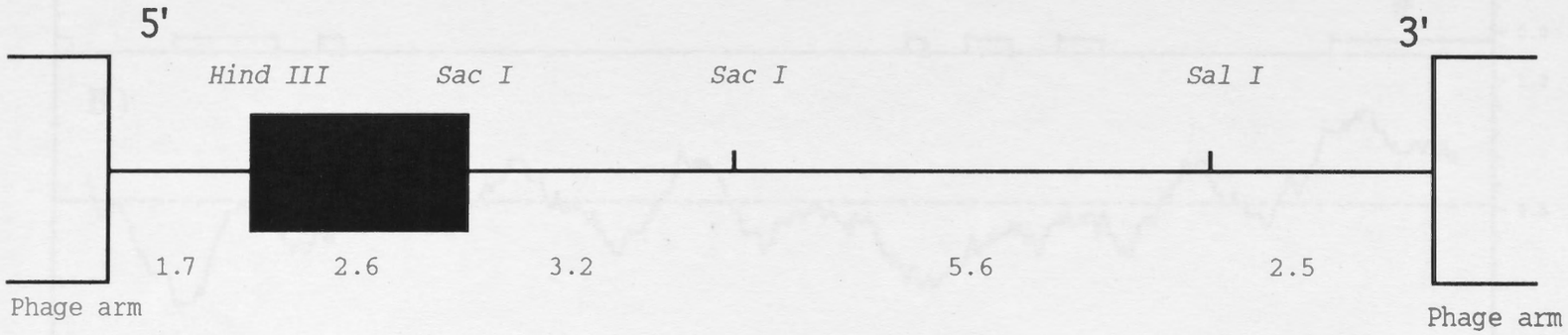


Figure 4.10; Map of the Hea-AT genomic phage clone. The location of the *Hind III/Sac I* sequence (Figure 4.4) is shown as a bold box. Numbers represent the size of each region of DNA between restriction enzyme sites (in kb). All *Hind III* sites are not shown.

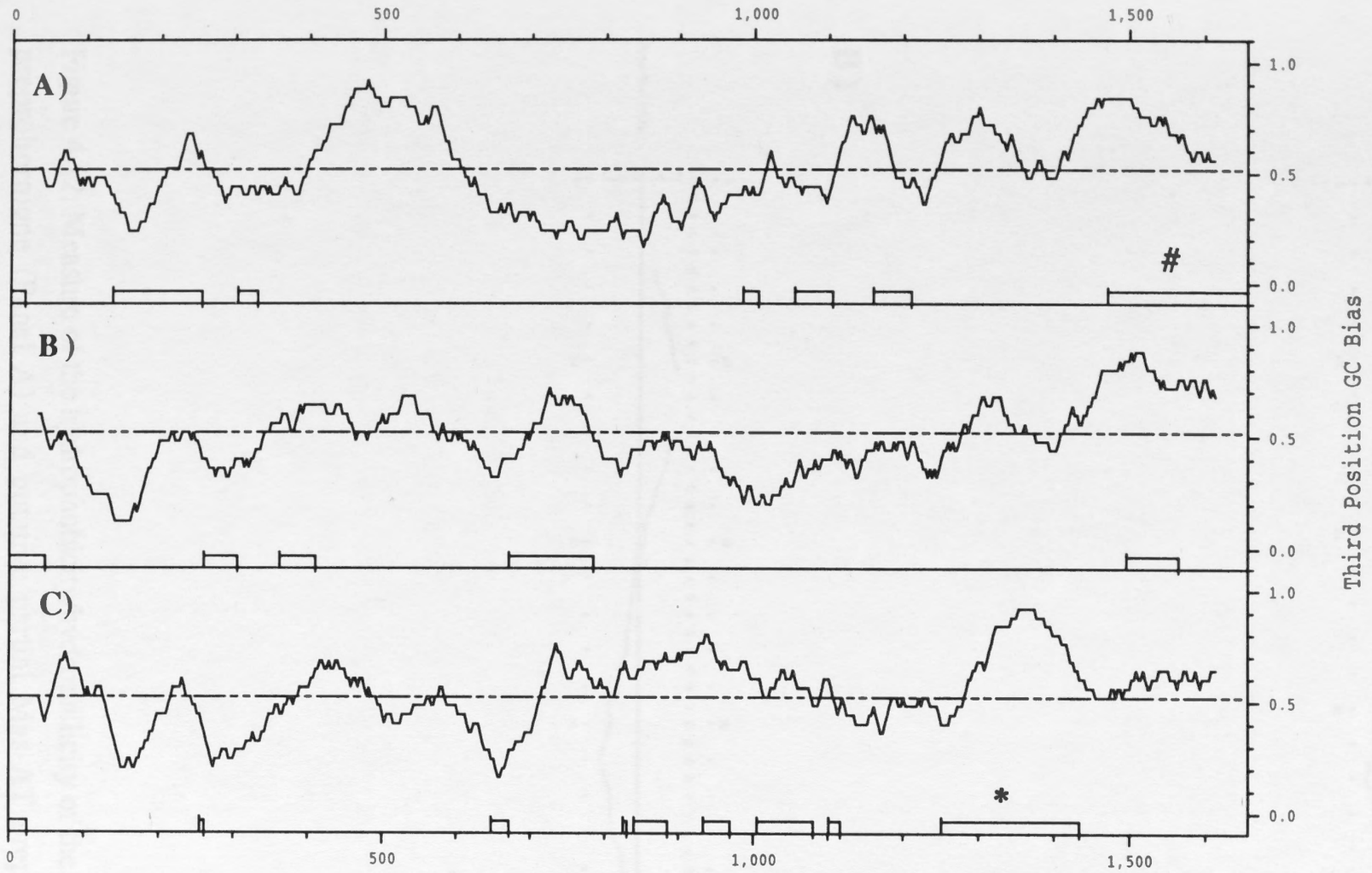
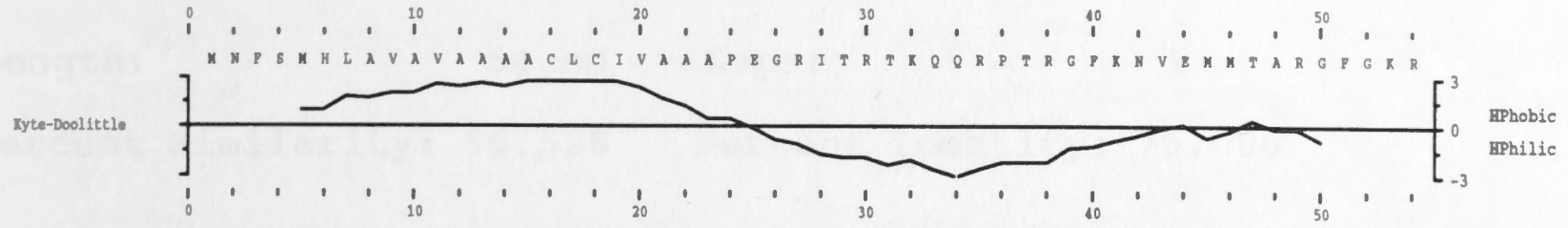


Figure 4.11; Analysis of the GC bias in the third codon position of the Hea-AT cDNA clone. Panel A, B and C represent the first, second and third reading frames respectively. At the bottom of each panel open reading frames are shown as rectangles. The region identified in Panel C (*) contains the putative Hea-AT open reading frame. The region identified in Panel A (#) is discussed in section 4.4.2 because it remains open at the end of the cDNA clone.

A)



B)

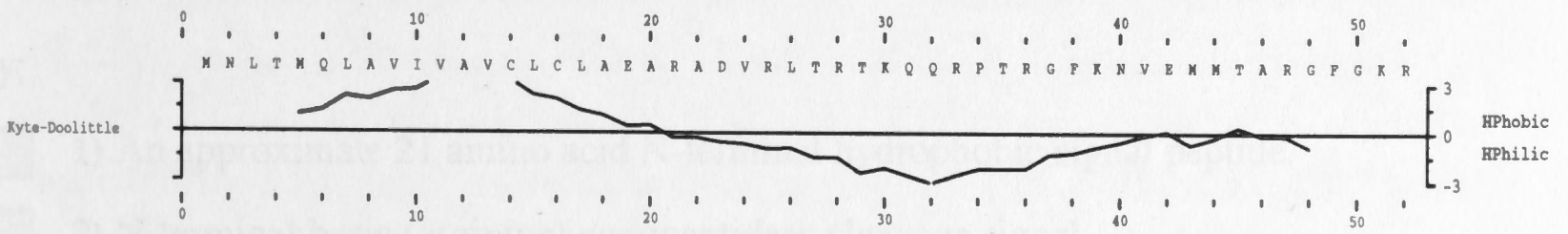
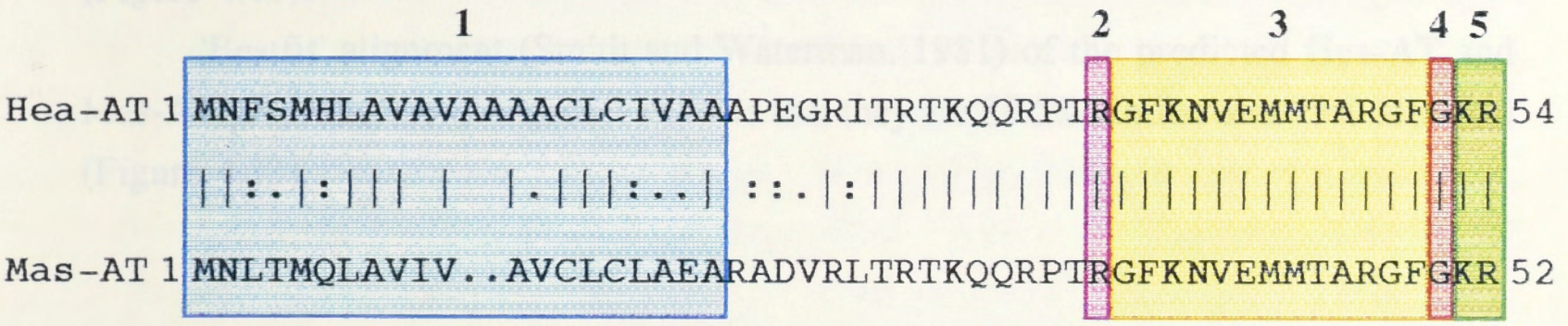


Figure 4.12; Measure of the hydrophobicity/hydrophilicity of the putative partial Hea-AT preprohormone (Panel A) and putative partial Mas-AT preprohormone (Panel B). Respective partial preprohormone sequences are presented above each graph.

```
Length:                54 bp     Gaps:                1
Percent Similarity: 86.538     Percent Identity: 75.000
```



Key;

1) An approximate 21 amino acid N-terminal hydrophobic signal peptide,

2) N-terminal basic (arginine) endopeptidase cleavage signal,

3) A single copy of the active peptide,

4) C-terminal α-amidation substrate (glycine),

5) C-terminal dibasic (lysine-arginine) endopeptidase cleavage signal.

Figure 4.13; Gap alignment and features of the putative N-terminal Hea-AT and Mas-AT preprohormone sequence.

methionine identified in Figure 4.3, through to the arginine residue most C-terminal to the Mas-AT peptide sequence (Figure 4.3). The sequence of this partial putative Mas-AT preprohormone is presented in Figure 4.12B and Figure 4.13.

Hydrophobicity analysis of the putative partial Mas-AT preprohormone structure shows that the 21 N-terminal amino acids are hydrophobic (Figure 4.12B) and are likely to be the hydrophobic signal sequence of this putative protein. Also present within the partial putative Mas-AT preprohormone are the potential α-amidation substrate and endopeptidase cleavage sites described above for the partial Hea-AT preprohormone (Figure 4.13).

'Bestfit' alignment (Smith and Waterman, 1981) of the predicted Hea-AT and Mas-AT preprohormone sequences shows that they are 86.5% similar and 75% identical (Figure 4.13).

4.4 DISCUSSION

4.4.1 Oligonucleotide Screening of Genomic Libraries

The first attempt to isolate the gene encoding the Hea-AT factor used Masat 1 as the oligonucleotide probe (section 4.3.1). Since the eight C-terminal amino acids of Mas-AT are essential for biological activity (Kataoka *et al.*, 1989) it was thought that this region of the peptide was most likely to be conserved between *M. sexta* and *H. armigera*. However, the C-terminal region contains a large number of amino acids encoded by multiple codons (Figure 4.1) which increases the degeneracy of the probe and consequently increases non-specific binding problems (Lathe, 1985; Sambrook *et al.*, 1989). Therefore, in light of the possible problems of using highly degenerate probes, a unique sequence oligonucleotide to the C-terminal end was used at low stringency conditions (Masat 1).

Comparison of the actual Hea-AT coding sequence (Figure 4.4) to that of Masat 1 shows that there are three mismatches out of 19, giving the oligonucleotide an estimated melting temperature (Tm) of 48.6°C (Bolton and McCarthy, 1962; Bonner *et al.*, 1973) with the target sequence. Thus, under the conditions used, Masat 1 should have hybridized with the putative Hea-AT gene (Figure 4.5). This may not have occurred because a target recombinant phage clone was not present in the pool screened, perhaps due to an underestimation of the *H. armigera* genome size. In each screening approximately 3 genome equivalents were screened, based on a genome size of $5x10^8$ bp for *He. virescens* (Martin Taylor, Department of Entomology, University of Arizona, U.S.A., personal communication). The fact that only one positive was obtained from the heterologous probing approach (section 4.3.3), instead of a predicted three, is consistent

with a larger genome size.

It appears to be pure chance that Masat 2 hybridized strongly to one of the ten clones isolated using Masat 1. Masat 2 had one mismatch to the target sequence (Figure 4.5) and a Tm of 54.6°C, thus it seems inconceivable that the nine clones discarded after the tertiary screening possessed the Hea-AT gene.

Following the negative result of the first library screening with unique sequence oligonucleotides the second attempt to isolate the Hea-AT gene utilized a highly degenerate oligonucleotide probe. In this instance a second oligonucleotide, designed from the N-terminal sequence was not used to counter-screen positive phage clones because it was uncertain how conserved the N-terminal sequence of the allatotropin was between *M. sexta* and *H. armigera*, since this region was not needed for Mas-AT biological activity in *M. sexta* (Kataoka *et al.*, 1989). However this approach only resulted in sequencing 10.5kb of non-target DNA (Table 4.1). The fears of screening with highly degenerate oligonucleotides were substantiated (Lathe, 1985; Sambrook *et al.*, 1989), with high amounts of background binding probably masking true positives.

Since screening attempts described above were unsuccessful, it was decided that an alternative route should be taken to isolate the Hea-AT gene. With the aid of Dr M. Greenstone (USDA), frozen eggs of the native North American moth, *M. sexta*, were obtained. DNA extracted from these eggs was used to construct a genomic library, the strategy being to isolate the Mas-AT gene from *M. sexta* and use this to isolate its homolog from the *H. armigera* genomic library.

When the *M. sexta* genomic library was screened with oligonucleotide probes (section 4.3.2), the regions of the Mas-AT peptide which may have been conserved throughout lepidopteran evolution no longer had to be predicted. This enabled library screening to occur with an oligonucleotide probe designed from the known N-terminal region of the peptide sequence which is comprised of amino acids encoded by codons of lower degeneracy (Masat 6). Counter-screening of the eleven positives obtained using Masat 6 (section 4.3.2) with an oligonucleotide probe designed from another region of the Mas-AT peptide (Masat 4a/c II) reduced the number of positives to three, all of which were found to come from the same region of the *M. sexta* genome (section 4.3.2).

In summary, I have found at least three important factors which need to be considered in the design of oligonucleotides for screening genomic libraries. Firstly, counter-screening of the positives with independent oligonucleotides can circumvent the sequencing of false positives. Secondly, oligonucleotides of up to 250 fold degeneracy give reasonable results with low background binding, whereas the use of oligonucleotides of more than 756 fold degeneracy begins to make the detection of true positives more difficult. As a corollary to the above, the chance of success when attempting to screen a genomic library of one species with oligonucleotides designed from

the protein sequence of another species from a different family, is strongly dependent not only on protein sequence conservation but also on the codon degeneracy of the target region(s).

4.4.2 Hea-AT Gene Structure

Comparison of the Hea-AT genomic and cDNA sequences indicates that the Hea-AT gene consists of at least three exons (Figure 4.7) which are referred to as the 5' exon, the Hea-AT coding exon and the 3' exon. During intron/exon splicing of mRNA the 3' end of the exon is known as the donor splice site and the 5' end of the exon is called the acceptor splice site. Therefore in the text below, the 5' extremity of the Hea-AT coding exon is referred to as the acceptor splice site and the 3' extremity of this exon is termed the putative donor splice site.

The finding that the putative donor splice site within the Hea-AT gene does not conform to the GT-rule (Figure 4.9) casts considerable doubt on whether the 240 downstream nucleotides of the Hea-AT cDNA clone represents part of the Hea-AT mRNA. Three possible explanations for the origin of the Hea-AT cDNA clone can be put forward, each discussed below.

Firstly, although not conforming to the GT-rule a donor splice site does exist within the Hea-AT gene at the region in question (Figure 4.7). Several exceptions to the GT-rule have been found (Shapiro and Senapathy, 1987). Of the approximate 1550 donor splice sites examined by Shapiro and Senapathy (1987), nine were found to defy the GT-rule. Five of these contained a GC instead of a GT, three a CT and one a CG (Shapiro and Senapathy, 1987), and in all these cases an adenine residue was found at positions +3 and +4 (as seen in Figure 4.9). Immediately upstream of the sequence examined for its homology with the consensus donor splice site (Figure 4.7) is a sequence similar to the donor splice site of a number of these genes with unusual donor splice sites;

AAG/**GC**AAGG	Human acetylcholine receptor (Shibahara *et al.*, 1985)
AAG/**GC**AAGG	Human superoxide dismutase (Levanon *et al.*, 1985)
AAG/**GC**AAGC	Chicken α-globin (Dodgson and Engel, 1983)
AAG/**GC**AAGC	Duck α-D globin (Erbil and Niessing, 1983)
AGG/**GC**AAGT	Soybean nodulin-24 (Katinakis and Verma, 1985)
TCG/**GC**AAGC	Hea-AT genomic clone (nucleotides 822-831, Figure 4.7).

However, the sequence homology between the Mas-AT and Hea-AT gene (Figure 4.5) extends 46 nucleotides further than the homology between the Hea-AT genomic and cDNA clones (Figure 4.7). These nucleotides can code for 11 amino acids which would

follow directly on the C-terminal end of the partial preprohormones (Figure 4.13) before a stop codon is reached in the Hea-AT gene sequence (Figure 4.4). These 11 amino acids are almost completely conserved (difference underlined) between *M. sexta* and *H. armigera*;

M. sexta; NH_2-AspArgProHis<u>Pro</u>ArgAlaGluArgGluPhe-COOH
H. armigera; NH_2-AspArgProHis<u>Thr</u>ArgAlaGluArgGluPhe-COOH

The conserved genomic sequence may play a role in intron/exon splicing. Such a role would seem unlikely because the consensus sequence of the acceptor site examined (Figure 4.8) extends much further into the intron sequence than that of the donor splice site consensus sequence (Figure 4.9). Although the homology detected by the 'Bestfit' program between the Mas-AT and Hea-AT genomic sequences (Figure 4.5) starts at the -3 position of the acceptor splice site although the consensus sequence for acceptor site splicing extends another 11 nucleotidesupstream. This indicates that the amino acid sequence given above may well be part of the Mas-AT and Hea-AT preprohormones.

A second possibility is that the Hea-AT cDNA clone has arisen from a misdirected splice event. A sequence can be found in the Hea-AT genomic clone approximately 20 nucleotides downstream of the putative donor splice site examined in Figure 4.9, which may also be a donor splice site;

NAG/**GT**AAGT Invertebrate donor consensus splice sequence (Figure 4.9)
AGC/**GT**AAGT Hea-AT genomic sequence (nucleotides 857-865, Figure 4.7).

If this were in fact a donor splice site, the amino acids DRPHTRAER would be part of the Hea-AT preprohormone and the question of homology between the Mas-AT and Hea-AT genomic sequences extending beyond the region of homology between the Hea-AT genomic and cDNA sequences would be solved. Another feature of the Hea-AT cDNA clone would also be clearer if the above sequence is a donor splice site. An open reading frame (frame 1, Figure 4.11) which extends to the end of the Hea-AT cDNA clone sequence may be in frame with the Hea-AT coding exon (frame 3, Figure 4.11), together producing a much larger open reading frame. However, if the partial open reading frame of frame 1 (Figure 4.11) is in fact part of the mRNA encoding the Hea-AT preprohormone it does not contain any sequences related to the Hea-AT peptide (data not shown).

The third possible explanation for the putative donor splice site not conforming to the GT-rule is that the Hea-AT cDNA clone has arisen from the ligation of two independent cDNAs during the construction of the cDNA library. An example of this has

previously been found in our laboratory. A 2.1kb FMRFamide cDNA clone was obtained from a *Lucilia cuprina* head cDNA library that contained a 270bp region of the FMRFamide gene, but the remaining 1.8kb of sequence was from an unknown gene (Sutherland and East, personal communication). If the Hea-AT cDNA clone is a hybrid clone of two unrelated cDNA then the open reading frame in frame 1 (Figure 4.10) could represent the coding sequence of another gene expressed in the pharate adult/adult *H. armigera* brain/retrocerebral complex. The translation of this open reading frame was used to search a database for homologous proteins, but none was found.

Of these three possible explanations for the failure of the putative splice donor site to comply with the GT-rule I believe the first is the most unlikely. This hypothesis relies on the Hea-AT having a very rare donor splice site as well as having an approximate 50 nucleotide sequence which is highly homologous to the Mas-AT genomic sequence that would be present within an intron. The third alternative presented is probably the most likely option because it would explain why the potential donor splice sites do not conform to the consensus sequence, as well as allow for the possibility that region of homology between the Mas-AT and Hea-AT gene sequences actually codes for part of the Mas-AT and Hea-AT preprohormones respectively. In addition an example of such a phenomenon has already been observed in the laboratory in which I have been studying.

With uncertainty about the validity of the putative 3' exon of the Hea-AT cDNA clone, conclusions about the complete structure of the Hea-AT gene are difficult to make. Clearly the Hea-AT gene consists of a minimum of two exons; the Hea-AT coding exon plus at least one 5' non-coding exon. During the construction of the cDNA library, random hexamers were used to prime first strand cDNA synthesis (section 4.2.8). In theory the reverse transcriptase enzyme once initiated will continue first strand cDNA synthesis until it reaches the 5' end of the mRNA molecule, and so the Hea-AT cDNA clone should contain the complete 5' untranslated leader sequence of the Hea-AT mRNA. Approximately 200bp of the 5' end of the Hea-AT genomic phage clone has been sequenced (data not shown) but did not contain any region of the Hea-AT cDNA clone. This observation combined with the fact that the *Eco RV* fragment of the Hea-AT cDNA, which encompassed the 5' extremity of the clone, failed to hybridize to the Hea-AT genomic phage clone, suggests that the 5' exon is at least 1.7kb upstream of the Hea-AT coding exon (Figure 4.10).

At least three possibilities exist for the structure of the 3' end of the Hea-AT gene. Firstly, it may well end at the stop codon identified in Figure 4.4. Secondly, if the 240 downstream nucleotides of the Hea-AT cDNA is part of the Hea-AT gene, then the intron is at least 11kb long, as this region of the cDNA clone does not hybridize to the Hea-AT genomic phage clone. Lastly, if an exon is present 3' of the Hea-AT coding exon, then the donor splice site must occur between the sequence coding for the peptide and the stop

codon presented in Figure 4.4. The best candidate for such a donor splice site has been identified above as extending from position 857-865 of the Hea-AT genomic clone (Figure 4.4).

Northern blot analysis using the Hea-AT cDNA clone as a probe was undertaken to characterize the mRNA size of the Hea-AT gene (data not shown). An approximate 1800 nucleotide mRNA region was detected, however the signal strength was extremely strong, producing an intense band during autoradiography within minutes. As the Hea-AT peptide is produced in no more than 22 cells in the brain/sub-oesophageal ganglion (section 5.3.1) this signal strength was difficult to rationalize. Subsequent realization that the cDNA was possibly a hybrid raised fears that rather than the Hea-AT mRNA being detected, another message from the same gene as the 240 downstream nucleotides of the cDNA clone was hybridizing to the Hea-AT cDNA clone. Therefore this experiment needs to be repeated without the 3' region of the cDNA clone.

A database search for sequences with homology to the Hea-AT cDNA clone identified a region on the complementary strand of the 5' untranslated sequence which has a high degree of homology to the 28S rRNA genes from many other species. A 'Bestfit' comparison of the *D. melanogaster* 28S gene with the complementary strand of the Hea-AT cDNA clone is presented in Figure 4.14. Similar results are observed for similar comparions with the mouse (Hassouna *et al.*, 1984) 79% over 325 nucleotides, rat (Chan *et al.*, 1983) 78% over 320 nucleotides and *Xenopus borealis* 28S rRNA genes (Ajuh *et al.*, 1991) 76 % over 315 nucleotides. It is therefore possible that the Hea-AT preprohormone is encoded from a genomic region that at least partially overlaps an rRNA gene(s) on the other strand. In theory, the hybrid cDNA clone hypothesis used as one explanation of the origin of the questionable 3' exon of the Hea-AT cDNA clone cannot be used to suggest that the 28S rRNA sequence has arisen through the hybridization of cDNA clones. This is because rRNA gene transcipts are not polyadenylated and should not have been retained during the isolation of poly A+ mRNA procedure (section 4.2.7).

4.4.3 Mas-AT and Hea-AT Preprohormone Structures

From the open reading frame of the Hea-AT cDNA clone the 54 N-terminal amino acids of the Hea-AT preprohormone can be determined (Figure 4.13). Sequence comparisons between the genomic sequence encoding the partial Hea-AT preprohormone and the sequence from the Mas-AT genomic clone (Figure 4.5) allows the putative 52 N-terminal amino acids of the Mas-AT preprohormone to be deduced (Figure 4.13). Both these partial preprohormones contain a region which is identical to the sequence of Mas-AT determined by Kataoka and co-workers (1989) indicating that the Hea-AT and Mas-AT peptide sequence has been completely conserved during evolution. Each of the partial preprohormone sequences possess the characteristics expected of neuropeptide

```
Length:                 319 bp    Gaps:                   5
Percent Similarity: 71.528    Percent Identity: 71.528


D. melanogaster 28S rRNA gene
    3322 CTGAATTTAAGCATATTAATTAGGGGAGGAAAAGAAACTAACAAGGATTT 3371
         | |||||||||||||||| | || ||||||||||||||||||| |||||||
     661 CCGAATTTAAGCATATTAGTAAGCGGAGGAAAAGAAACTAACCAGGATTT 612
H. armigera cDNA


    3372 TCTTAGTAGCGGCGAGCGAA.AAGAAAACAGTTCAGCACTAAGTCACTTT 3420
         |||||||||||||||||||| | |||   ||  ||||||| | || |
     611 CCTTAGTAGCGGCGAGCGAACAGGAATGAAGCCCAGCACTGAATCCCGCC 562

    3421 GTCTATATGGCAAATGTGAGATGCAGTG.TATGGAGCGTCAATATTCTAG 3469
         ||   |  |||    | ||||||  ||| |  ||||  ||    |||| |
     561 GTTGTTCAGGC.GGCGGGAGATGTGGTGTTCGGGAGGTTCCGCTTTCTCG 513

    3470 TATGAGAAATTAACGATTTAAGTCCTTCTTAAATGAGGCCCGTATAACGT 3519
         |  | |       |  |  |||| | |||| || | ||||  | |  ||
     512 T.CGCGGTCGCTCCTGTCCAAGTTCGTCTTGAACGGGGCCGTTTTCCCGC 464

    3520 TAATG.........................ATTACTAGATGATGTTTC 3542
           | |                        |  | ||| |   | ||
     463 AGAGGGTGCCAGGCCCGTAGCGACGGAGGACCGTGGCGAGAGGGACTCTC 414

    3543 CAAAGAGTCGTGTTGCTTGATAGTGCAGCACTAAGTGGGTGGTAAACTCC 3592
         |  ||||||| |||||||| |||||||| ||||||||||||||||||||
     413 CTTAGAGTCGGGTTGCTTGAGAGTGCAGCCCTAAGTGGGTGGTAAACTCC 364

    3593 ATCTAAAACTAAATATAAC 3611
         |||||| |||||||| ||
     363 ATCTAAGGCTAAATATTAC 345
```

Figure 4.14; 'Bestfit' comparison of the *D. melanogaster* rRNA gene complex (Tautz *et al.*, 1988) with the complementary strand of the Hea-AT cDNA clone. A 319bp region of the *D. melanogaster* 28S rRNA gene is 72% similar to the minus strand of the 5' region of the Hea-AT cDNA.

preprohormones (section 1.4, Figure 4.13).

Hydropathy analysis of the deduced polypeptides indicates that about 25 N-terminal amino acids in the *H. armigera* sequence and 21 N-terminal amino acids in the *M. sexta* sequence form regions which are hydrophobic in nature (Figures 4.12A and B, respectively). These two regions of the putative preprohormones are plausible N-terminal signal peptides (section 1.4.1), commonly observed to be 20-30 amino acids long (Sossin *et al.*, 1989). The precise site of signal peptide cleavage can only be determined experimentally, however it is predicted to occur at amino acid 22 (Figure 4.13) for the Hea-AT preprohormone according to the von Heijne (1986) algorithm (section 1.4.1, data not shown). The algorithm could not accurately predict this site in the Mas-AT preprohormone, which apparently belongs to the 20-25% of sequences whose signal peptide cleavage site cannot be determined by this method (von Heijne, 1986).

In the both partial preprohormone sequences the allatotropic peptide is flanked by putative endopeptidase cleavage sites (section 1.4.2, Figure 4.13). At the C-terminal end a basic Lys-Arg sequence is present which is the most common recognition site for the action of specific prohormone processing endopeptidases. At the N-terminal end of the active peptide a single Arg residue is found, the most common single amino acid recognition site for endopeptidase enzymes. The peptide sequence surrounding this single Arg complies with recent hypotheses on the rules governing cleavage at such sites (Devi, 1991; Nakayama *et al.*, 1992 (section 1.4.2);

1) another arginine residue is found three amino acids to the N-terminal end,
2) no cysteine is present close to the putative cleavage site,
3) no aromatic amino acid is present just N-terminal to the putative cleavage point
4) no hydrophobic aliphatic residue is present just C-terminal to the putative cleavage point.

The biological activity of the Mas-AT is dependent on C-terminal amidation (Kataoka *et al.*, 1989), the template for which is invariably a glycine (section 1.4.3). The putative Mas-AT preprohormone has a glycine residue situated between the C-terminal end of the active peptide and the dibasic Lys-Arg endopeptidase cleavage substrate. Such an α-amidation substrate is also observed in the putative preprohormone structure of the Hea-AT, strongly suggesting this peptide is amidated as well.

Neuropeptide preprohormones often code for multiple copies of the same peptide or related peptides (see section 1.4). There is only one copy of Hea-AT within the putative preprohormone as presently defined. There is complete amino acid sequence conservation between the putative Mas-AT and Hea-AT proteins from the threonine residue (position 29 for *H. armigera*; 27 for *M. sexta*) to the C-terminal end of the Mas-AT sequence (Figure 4.13). This conservation may be due to the presence of other

biologically active peptides. Based on the rules of single arginine endopeptidase cleavage sites (section 1.4.2), the endopeptidase which putatively cleaves at the N-terminus of the Mas-AT/Hea-AT active peptides could also theoretically cleave the prohormones at one other site upstream of the Mas-AT/Hea-AT peptide, producing a conserved peptide (TKQQRPT) which has no sequence homology to any reported peptides.

4.5 SUMMARY

Oligonucleotide probes designed from the Mas-AT peptide sequence were used to isolate a region from the *M. sexta* genome which putatively encodes this peptide. The Mas-AT coding region was then used to clone the homologous region from the *H. armigera* genome which was used in turn, to isolate a cDNA clone encoding the Hea-AT peptide.

Alignment of the Hea-AT genomic sequence with the Hea-AT cDNA sequence suggests that the Hea-AT gene consists of at least three exons. Analysis of the putative acceptor splice site within the Hea-AT genomic sequence showed that it complied with the AG-rule of acceptor splice sites and confirmed that an exon(s) 5' of the Hea-AT coding exon exists. However, analysis of the putative donor splice site within the Hea-AT genomic sequence showed that it did not conform with the GT-rule of such splice sites. This casts considerable doubt on both the existence of a 3' exon in the Hea-AT gene and on the origin of the 240 downstream nucleotides of the Hea-AT cDNA clone.

The 54 N-terminal amino acids of the Hea-AT preprohormone have been characterized. The deduced protein contains an N-terminal region with the properties of an hydrophobic signal sequence, a single copy of the Hea-AT peptide and a glycine residue immediately C-terminal to the Hea-AT peptide which could act as a substrate for α-amidation. The Hea-AT peptide (plus C-terminal glycine) is flanked by an arginine on the N-terminal side and a lysine-arginine on the C-terminal side which putatively act as recognition sites for endopeptidase cleavage to liberate the Hea-AT peptide from the prohormone.

Molecular biology techniques have determined the Hea-AT peptide to be identical to the Mas-AT peptide isolated by Kataoka and co-workers (1989).

CHAPTER 5

SITES OF Hea-AT PEPTIDE SYNTHESIS AND CORPUS ALLATUM ULTRASTRUCTURE IN VIRGIN FEMALE *Helicoverpa armigera*

CHAPTER 5

5.1 INTRODUCTION

Traditionally the identification of brain neurosecretory cells (NSC) involved in the regulation of JH synthesis has relied upon experiments which denervate the CA, destroy neurosecretory areas of the brain, or electrostimulate areas of the brain, and then examine the subsequent effects on JH production (Tobe and Stay, 1985; Khan, 1988). Such work has been complemented with neuronal tracing studies, using substances such as cobalt chloride and horseradish peroxidase, which allow the axonal paths of nerves innervating the CA to be identified (Khan, 1988). More recently, the structural characterization of JH regulatory peptides (Figure 1.5) has allowed the application of immunohistochemical techniques to determine the source, axonal pathways and sites of release of allatotropins, allatostatins and allatinhibins. This technology has been used to map the sites of production and release of other neuropeptides including; the diuretic hormone (Veenstra and Hagedorn, 1991), eclosion hormone (Copenhaver and Truman, 1986a) and prothoracicotropic hormone (O'Brien *et al.*, 1988) in *M. sexta*.

The NSC controlling the inhibition of JH synthesis in virgin adult female *D. punctata* have clearly been identified. Denervation of the nervi corpori cardiaci I (NCC I) and destruction of medial NSC (MNSC) both resulted in an increase in JH synthesis from the CA of virgin *D. punctata* (Ruegg *et al.*, 1983). Nickel chloride backfilling experiments showed that axons of the NSC of the pars intercerebralis (MNSC) innervate the cockroach CA via the NCC I, suggesting that NCC I denervation and MNSC destruction are preventing the same inhibitory factor(s) from reaching the CA. Destruction of lateral NSC (LNSC) also resulted in gland stimulation, but severance of the NCC II, which is the pathway by which NSC axons from LNSC reach the CA, did not alter CA activity as measured in a RCA. Axons of the pars intercerebralis and pars lateralis were observed to overlap by backfilling experiments, which led the authors to suggest that the LNSC stimulate the release of allatostatin from MNSC axons (Ruegg *et al.*, 1983). Recent immunohistochemical work appears to contradict the axonal backfilling and denervation experiments of Ruegg and co-workers (1983). An antibody against the *D. punctata* allatostatin 1 (Dip-ASA1) (Figure 1.5), was strongly immunoreactive with two pairs of cells in the lateral region of the adult female *D. punctata* protocerebrum (Stay *et al.*, 1992). The immunoreactivity could be traced from this lateral region of the brain to the CA via the NCC II. In light of these data it was hypothesized that the MNSC mediate allatostatin release from LNSC (Stay *et al.*, 1992) and not vice versa as described by

Ruegg and co-workers (1983). If the LNSC are considered to be the site of allatostatin synthesis, then the severance of the NCC I may prevent allatostatin release, whereas severance of the NCC II did not stop allatostatin reaching the CA through the hemolymph (Stay *et al.*, 1992).

In adult female lepidopterans very little is known about the cells in which allatotropin is produced and how it reaches the CA. Copenhaver and Truman (1986b) used cobalt chloride backfilling to identify NSC from the medial and lateral regions of the protocerebrum which innervate the CA of adult female *M. sexta* (Figure 1.1), but their role in JH regulation, if any, is undetermined. Severance of the fused NCC I/II at the time of adult female eclosion in *M. sexta* greatly reduced egg production (Sasaki and Riddiford, 1984; Ishizaka *et al.*, 1987), suggesting that the Mas-AT may reach the CA via the NCC I/II. Experiments involving ablation of MNSC of adult female *H. zea* resulted in a considerable loss of CA activity (Satyanarayana *et al.*, 1991), but this does not necessarily mean that *H. zea* allatotropin is produced in this region. *H. zea* MNSC may be like those in virgin adult female *D. punctata* and just regulate JH release (see above), or MNSC ablation may have destroyed axons originating from other regions of the brain which project through the pars intercerebralis to the CA for release of allatotropin.

This chapter describes preliminary attempts to identify sites of allatotropin synthesis and release in the brain/retrocerebral complex of adult female *H. armigera* by immunohistochemistry with a polyclonal antiserum against the Mas-AT peptide. Some basic ultrastructural characterization of biologically active CA from a newly eclosed female moth has been carried out to complement the immunohistochemical studies.

5.2 MATERIALS AND METHODS

5.2.1 Immunohistochemistry

Brain/retrocerebral complexes were dissected from pharate adult and newly eclosed adult female *H. armigera* as previously described (section 2.2.2) and fixed in 4% (w/v) paraformaldehyde in PBS (PBS; 8mM Na_2HPO_4, 20mM NaCl, 2.7mM KCl and 1.3mM KH_2PO_4, adjusted to pH 7.2). Tissues were permeabilized by successive incubations in; 70% methanol in PBS (5 min), 100% methanol (60 min), 70% methanol in PBS (5 min), PBS (2 x 5 min), followed by two 5 min washes in PBT (PBS + 0.1% v/v Triton X-100). After a 30 min incubation in PBT+N (PBT + 5% normal goat serum) to block non-specific immunoglobulin binding, the brain/retrocerebral complexes were incubated in a 1:1000 dilution (in PBT+N) of a rabbit polyclonal Mas-AT antiserum (a gift of the Sandoz Corp., Palo Alto, USA) for 2 hours at room temperature followed by 40 hours at 4°C.

Unbound primary antibody was removed from the tissue by several washes in PBT, the tissue re-blocked with a 30 min incubation in PBT+N and incubated in a 1:40 dilution (in PBT+N) of a fluorescein iso-thiocyanate conjugated sheep anti-rabbit secondary antibody (Silenus) for 2 hours at room temperature followed by 24 hours at 4°C. Unbound secondary antibody was removed by several washes in PBT, and the tissue mounted on to microscope slides in 0.01% (w/v) *p*-phenylenediamine (Sigma) in 70% glycerol.

Immunoreactivity was visualized using a confocal laser scanning microscope (Leica) which utilizes a computer regulated laser beam to produce digital images of the tissue at selected intervals. This technology allows an accurate, high resolution, three dimensional image to be obtained without tissue sectioning (Hines *et al.*, 1993).

The brain of adult female *H. armigera* is over 220μm thick (from front to back) and could not be completely imaged from a single surface in the Leica microscope, which can optically section only to a depth of 170μm. In addition, there was substantial quenching of the fluorescent signal when cells were observed through more than 100μm of tissue. Therefore images from both anterior and posterior aspects of the brain were obtained.

Twelve brain/sub-oesophageal complexes from either pharate adult (4 preparations) or newly eclosed adult (less than a day old) moths were examined. Tissue damage during dissection or subsequent removal of tracheal material prevented complete data from being obtained for all 12 specimens. All immunoreactive cells described below were observed in at least 6 preparations. Five intact samples were imaged from both anterior and posterior surfaces. For these preparations the tissue was optically sectioned in 25 slices, each 5μm thick. Image analysis software was then used to reconstruct the digitised data to provide a single picture containing all information for that field of view. With a 10x objective it was possible to analyse the entire surface of a brain/sub-oesophageal ganglion complex in a set of six partially overlapping images. Montages prepared from these pictures were used to map sites of Mas-AT immunoreactivity.

5.2.2 Electron Microscopy

CA were excised from newly eclosed adult female *H. armigera* under fixative (2% glutaraldehyde in 0.05M cacodylate buffer (pH 7.2) containing 5% sucrose) and then incubated in the fixative at 4°C for 2 hours. Tissues were washed in several changes of 0.05M cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide (in 0.05M cacodylate buffer, pH 7.2). CA were dehydrated in ethanol, embedded in epoxy resin (Spurr, 1969), sectioned, and then stained with alcoholic uranyl acetate and lead citrate (Reynolds, 1963). Sections were examined using a JEOL 100C electron microscope at 80kV.

5.3 RESULTS

5.3.1 Mas-AT Immunoreactivity in the Brain

The wholemount technique does not allow accurate identification of the different regions of the brain, and description of the positions of Mas-AT immunoreactive cells will therefore refer only to the major divisions; the protocerebrum, deutocerebrum and tritocerebrum. The majority of the NSC that innervate the retrocerebral complex are located in the superior protocerebrum in regions commonly referred to as the pars intercerebralis and pars lateralis (Khan, 1988). In adult lepidopterans the axons from these neurosecretory centres leave the brain as the nerve bundle called the NCC I/II (Figure 1.1). The deutocerebrum comprises the antennal centres and is primarily responsible for olfaction, while the tritocerebrum partially controls feeding behaviour and also contains NSC that convey material to the retrocerebral complex via the NCC III. The circum-oesophageal connectives that link the brain to the sub-oesophageal ganglion and ventral nerve cord are highly condensed, with the boundary between the tritocerebrum and sub-oesophageal ganglion not readily apparent in adult lepidopterans. Nevertheless, the region ventral and ventro-lateral to the oesophageal foramen clearly belongs to the sub-oesophageal ganglion.

No consistent differences in the pattern of immunoreactivity were observed between complexes from pharate adults and newly eclosed adults. Results from the five preparations that were completely analysed are summarized diagrammatically in Figure 5.1. Immunoreactive perikarya were observed in five distinct regions, each occurring on both left and right sides of the brain with a bilateral symmetry. Region 1 (Figure 5.1A and Figure 5.2) contained 6 strongly immunoreactive cells located in the pars lateralis of the superior protocerebrum. These cells were clearly visible from both the anterior and posterior surface and were juxtaposed to an area of intensely labelled neuropile that probably corresponds to the calyx of the mushroom body (Figure 5.1A and 5.3). A group of 6 or 7 smaller cells (10-15μm in diameter) were medially located on the posterior surface of the protocerebrum, slightly dorsal to the oesophageal foramen (Region 2, Figure 5.1B and Figure 5.4). These cells were labelled with variable intensity in different preparations. The 4 cells in Region 3 were located on the posterior surface of the protocerebrum at approximately the same level as those of Region 2, but in a more lateral position (Figure 5.1B and 5.5). Region 4 comprised a pair of strongly labelled cells positioned laterally in the tritocerebrum (Figure 5.1B and 5.6). The fifth group of cells were a bilaterally symmetric set of 3 pairs, located medially in the ventral region of the sub-oesophageal ganglion (Region 5, Figure 5.1A and Figure 5.7). Extensive

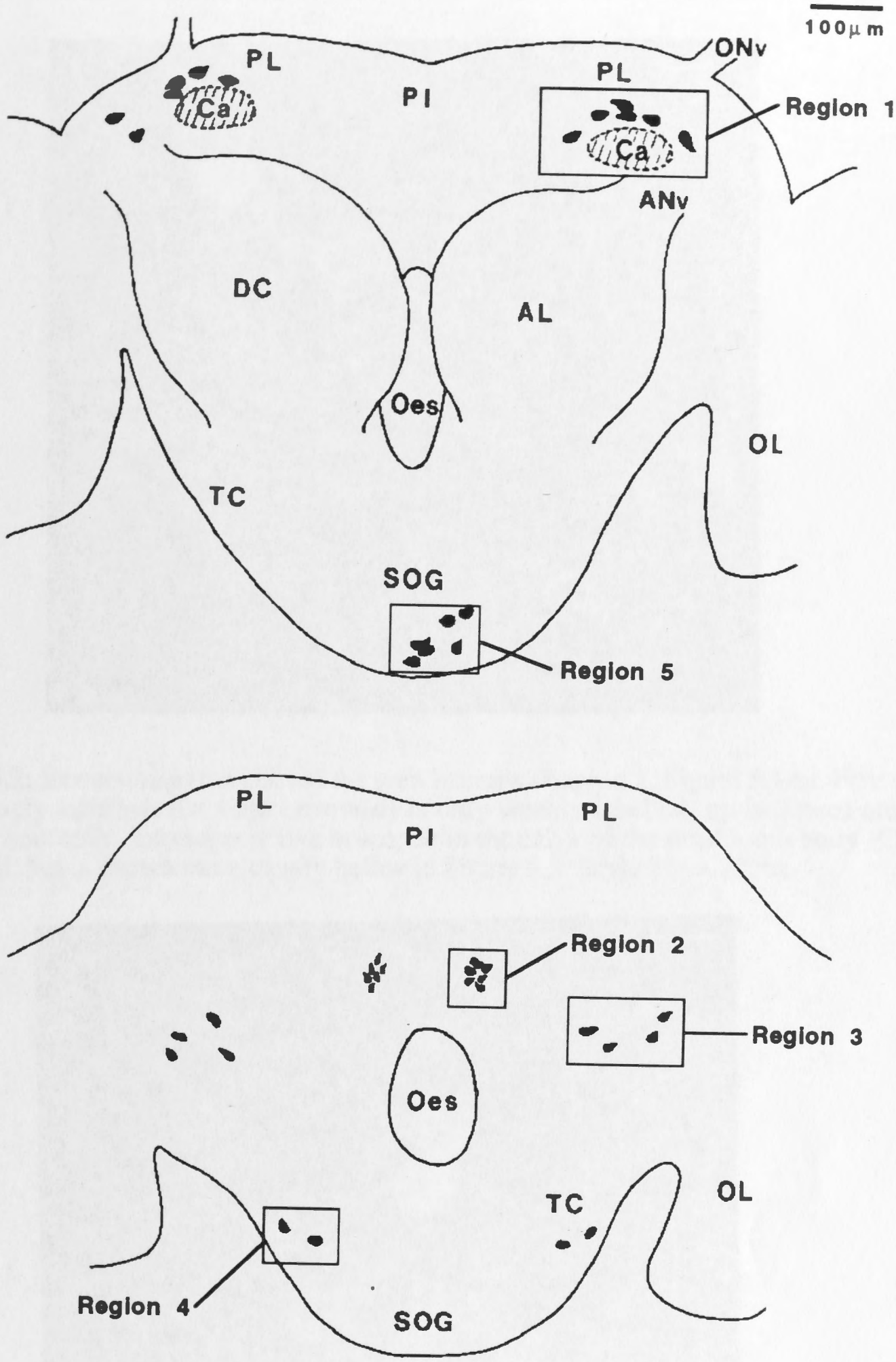


Figure 5.1; Summary of the immunohistochemical localization of Mas-AT immunoreactivity in the brain and sub-oesophageal ganglion of adult female *H. armigera*. A) Cells in the anterior brain, viewed from the anterior aspect. B) Cells in the posterior brain, viewed from the posterior surface. The boxed regions 1 to 5 indicate the sites and numbers of immunoreactive perikarya described in the text. The shaded areas represent regions of intense immunoreactivity which may be in the calyces of the mushroom body. Scale Bar = 100µm.

ABBREVIATIONS: AL, antennal lobe; ANv, antennal nerve; Ca, calyx of mushroom body; DC, deutocerebrum; Oes, oesophageal foramen; OL, optic lobe; ONv, ocellar nerve; PI, pars intercerebralis; PL, pars lateralis; SOG, sub-oesophageal ganglion; TC, tritocerebrum.

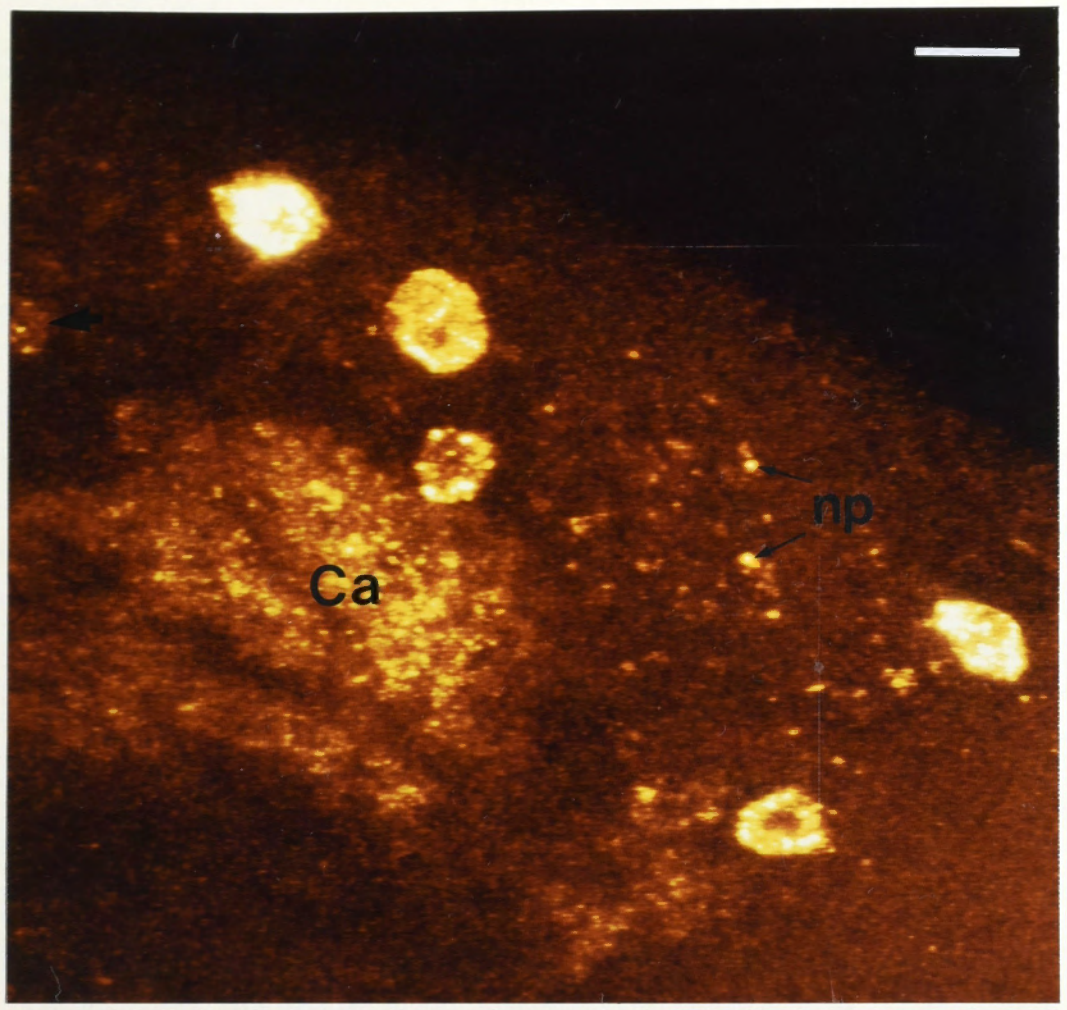


Figure 5.2; Immunoreactive cells in the pars lateralis (Region 1, Figure 5.1A). Five cells are strongly labelled; the sixth (arrowed) is only weakly labelled. np indicates area of labelled neuropile. Immunoreactive neuropile in the calyx of the mushroom body (Ca) is indicated, but is shown more clearly below in Figure 5.3. Scale bar = 20μm.

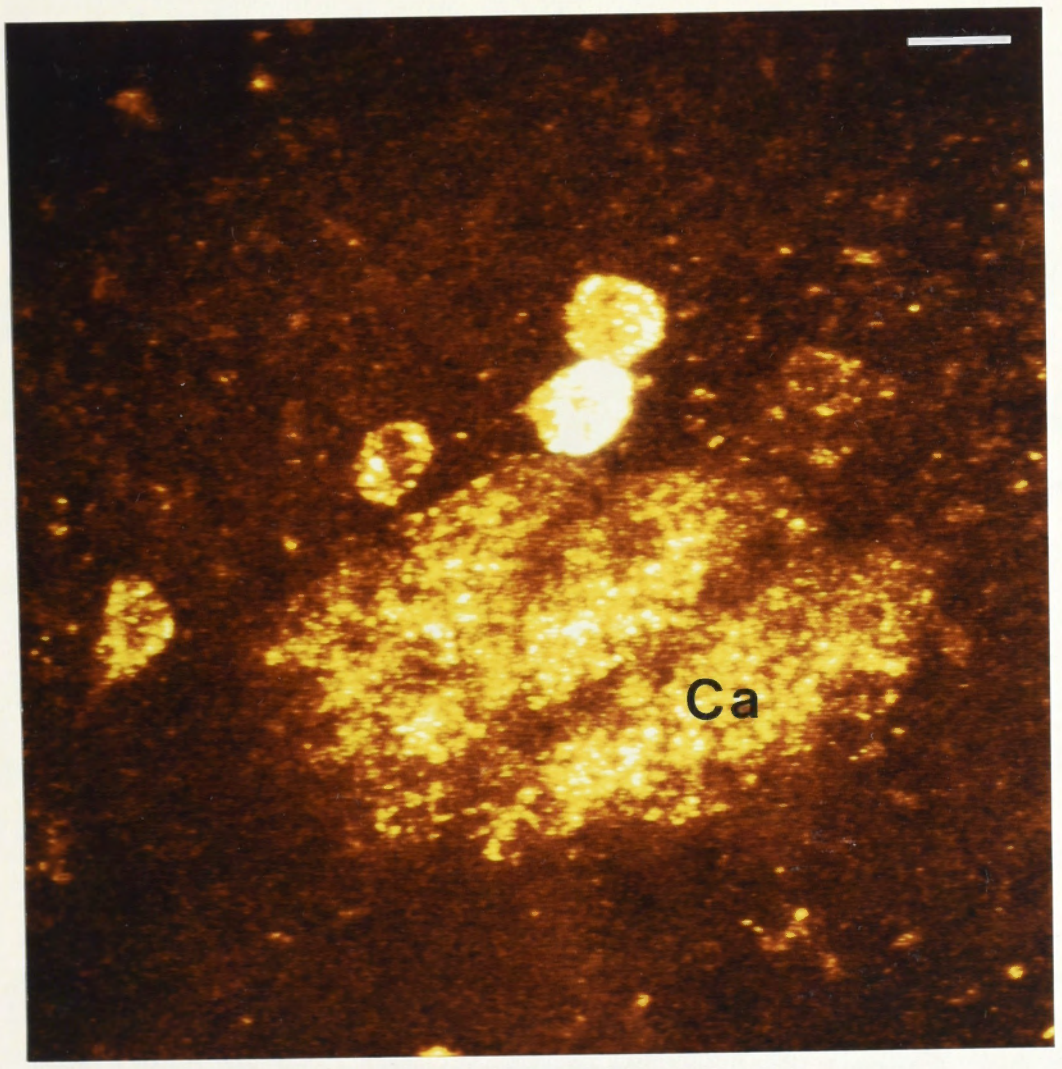


Figure 5.3; Immunoreactive cells in Region 1 situated dorsal to an area of intensely labelled neuropile (Ca) that is probably in the calyx of the mushroom body. Scale Bar = 20μm.

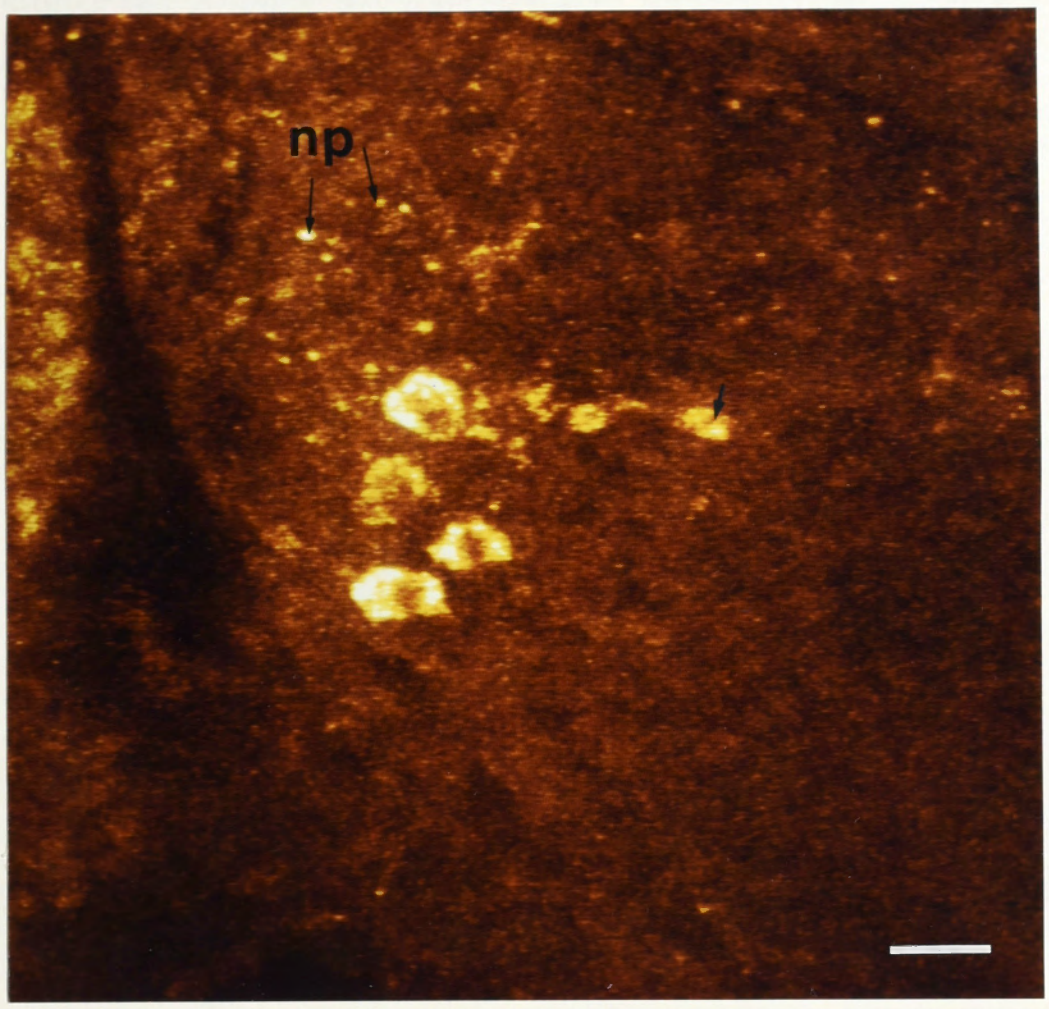


Figure 5.4; Five cells in Region 2 of the posterior protocerebrum (Figure 5.1B). Four cells are clearly visible; the fifth (arrowed) is only partially visible in the reconstruction. Note also extensive immunoreactive neuropile (np) in this region of the brain. Scale Bar = 20μm.

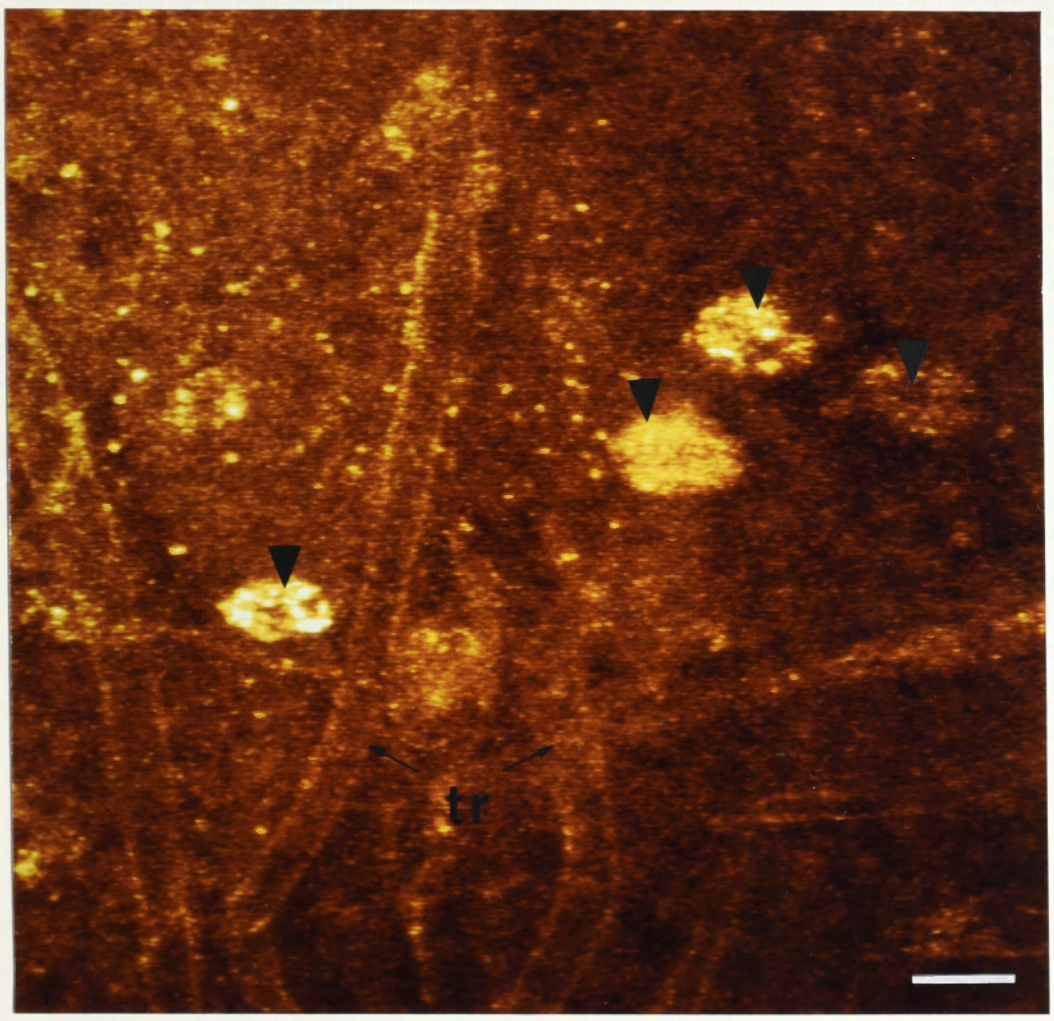


Figure 5.5; The four immunoreactive perikarya (arrowheads) of Region 3 (Figure 5.1B); tracheae (tr) on the posterior surface of the brain. Scale Bar = 20μm.

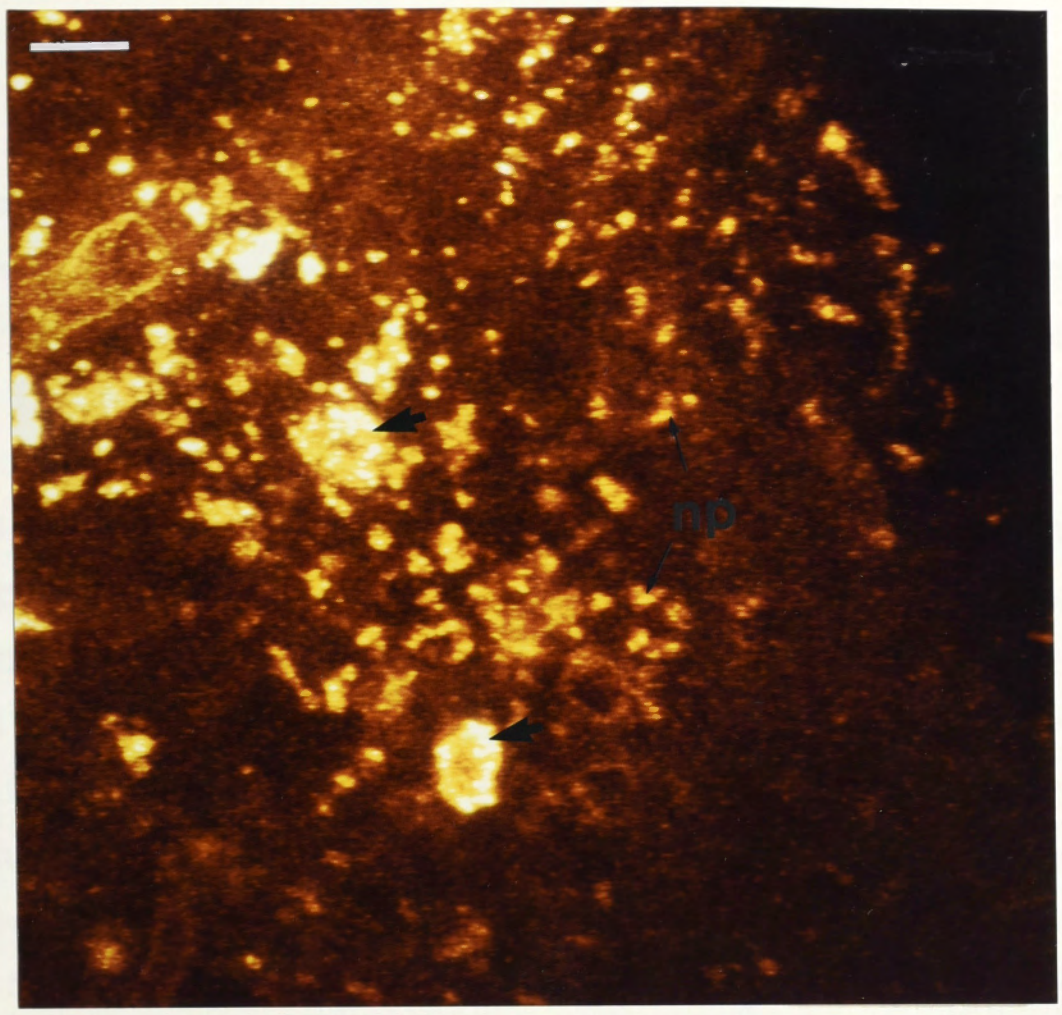


Figure 5.6; Two immunoreactive cells in the posterior lateral tritocerebrum (Region 4, Figure 5.1B), situated in a region of immunoreactive neuropile (np). Scale Bar = 20μm.

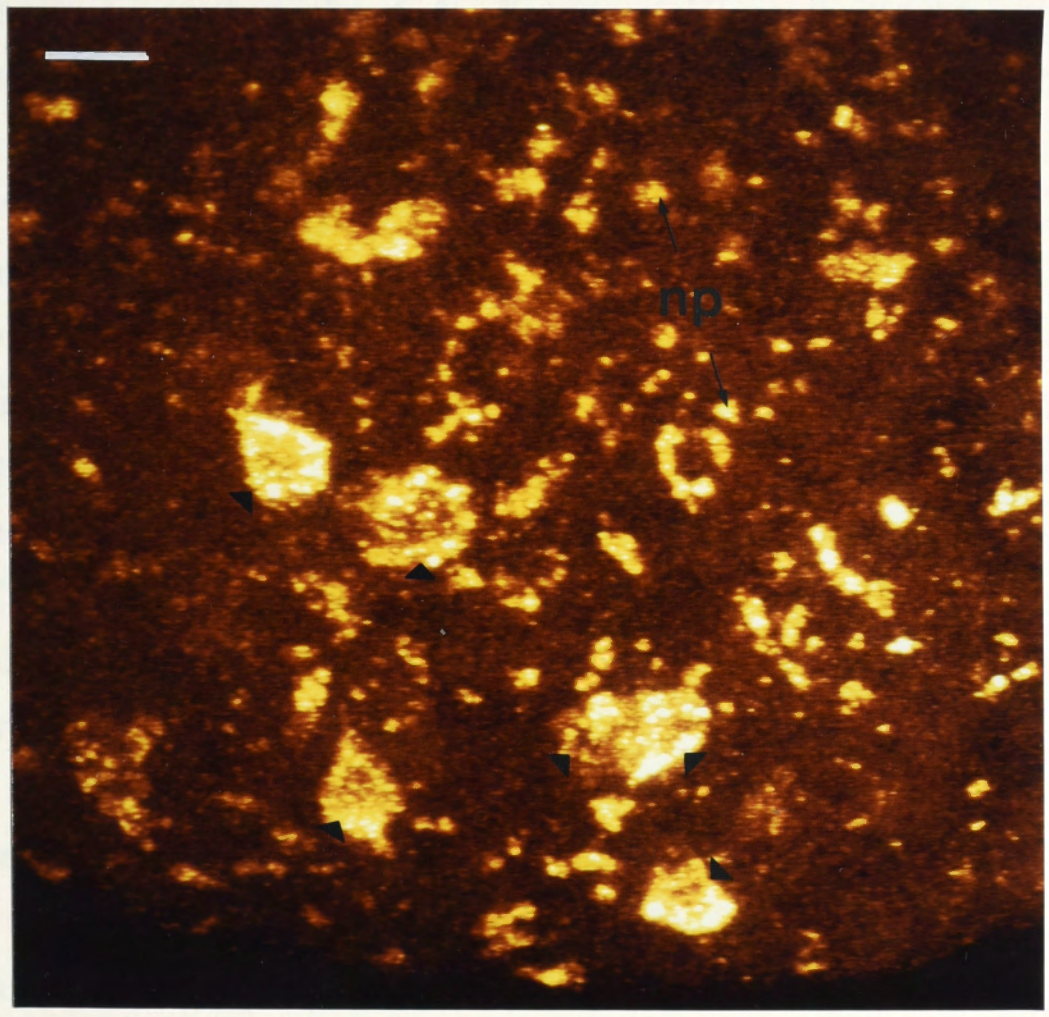


Figure 5.7; Immunoreactive cells (arrowheads) of the sub-oesophageal ganglion (Region 5, Figure 5.1A) seen from the anterior surface. Neuropile (np) of the sub-oesophageal ganglion contained extensive Mas-AT immunoreactivity. Scale Bar = 20μm.

immunoreactivity was evident in the neuropile of the protocerebrum (Figure 5.2 and 5.4), the tritocerebrum (Figure 5.6) and the sub-oesophageal ganglion (Figure 5.7), but it was not possible identify the cells responsible for the neuropile immunoreactivity in this study.

5.3.2 Mas-AT Immunoreactivity in the Retrocerebral Complex

The occurrence of Mas-AT immunoreactive material in the retrocerebral complex was examined in the same 12 moths used to map sites of immunoreactivity in the brain. In all cases immunoreactivity was noted in the CA, but the amount varied considerably between different individuals. Staining intensity was comparatively low in the CA of pharate adults (Figure 5.8) and was fairly consistent between glands for the four individuals examined. Results were more variable for newly eclosed moths; some glands resembled those of pharate adults, while others (Figure 5.9) contained much greater amounts of immunoreactivity. The glands of newly eclosed moths were much larger than those of pharate adults (Figures 5.8 and 5.9), but among newly eclosed individuals the amount of immunoreactivity did not appear to be correlated to gland volume.

The optical sectioning and three-dimensional image analysis capabilities of the confocal microscope allowed closer examination of the distribution of Mas-AT immunoreactivity within the CA. Figure 4.10 is a stereo-pair showing that immunoreactivity is distributed throughout the CA, presumably in nerve terminals that extend into the tissue between the endocrine cells (section 5.3.3).

There was no evidence of Mas-AT immunoreactivity in the CC (Figure 5.11). Weak staining was observed in axons of the NCC I/II but not in the NCC III (Figure 5.11). Although immunoreactivity in the NCC I/II is very weak it was observed several times in different preparations. The identification of nerves was based on optical sectioning and three-dimensional reconstruction. All specimens were mounted with the dorsal surface of the tissue uppermost. In this position the NCC I/II lies above and medial to the NCC III. No immunoreactivity was ever observed in other potential neurohemal tissues, such as the aorta, associated with the retrocerebral complex, suggesting that the nerves terminating within the CA are the only sites of peptide release.

5.3.3 *H. armigera* CA Ultrastructure

At the start of this study there was no information on CA structure or gland innervation in *Helicoverpa sp.* and relatively little for any other lepidopteran species. Although a detailed analysis was beyond the scope of this project, a small study of CA ultrastructure was made to provide additional information on gland innervation, to complement the results obtained from immunohistochemistry.

Figure 5.12 is a low power transmission electron micrograph of the periphery of

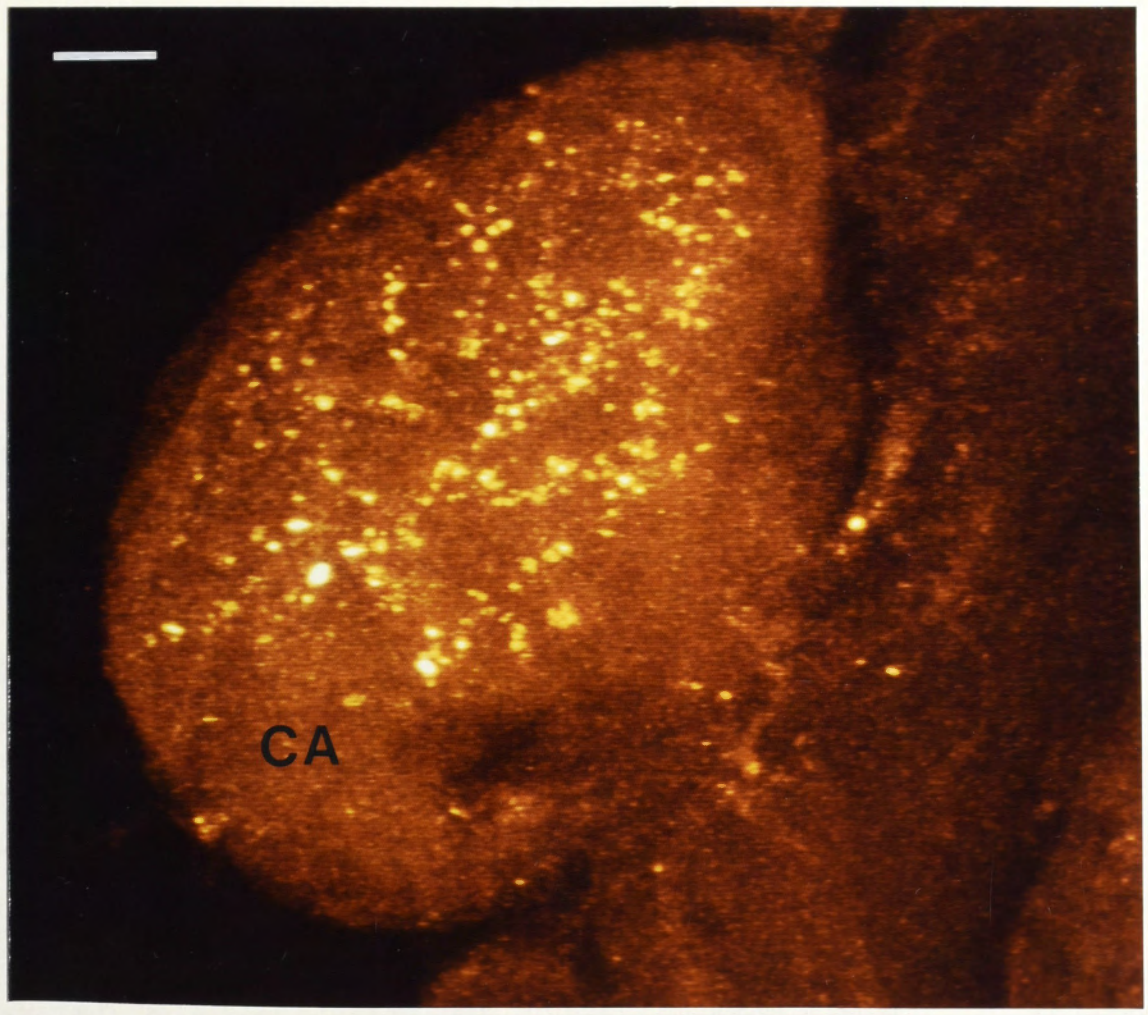


Figure 5.8; Corpus allatum from pharate adult *H. armigera* showing Mas-AT immunoreactivity uniformly distributed throughout the gland. Scale Bar = 20μm.

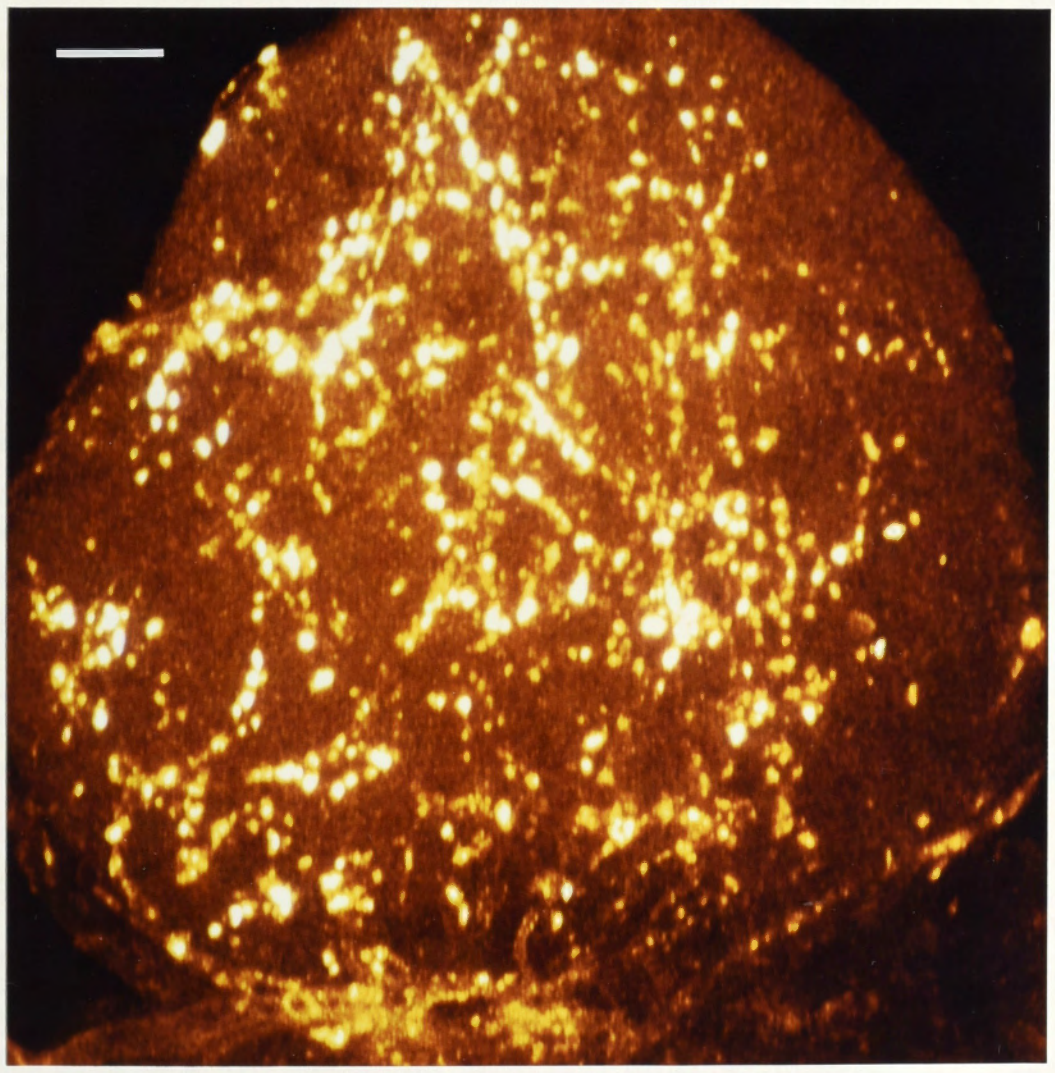


Figure 5.9; Corpus allatum from newly eclosed adult *H. armigera* with a large amount of Mas-AT immunoreactivity distributed in the nerves investing the gland. Note the increased size of the gland compared to that of the pharate adult (Figure 5.8). Scale Bar = 20μm.

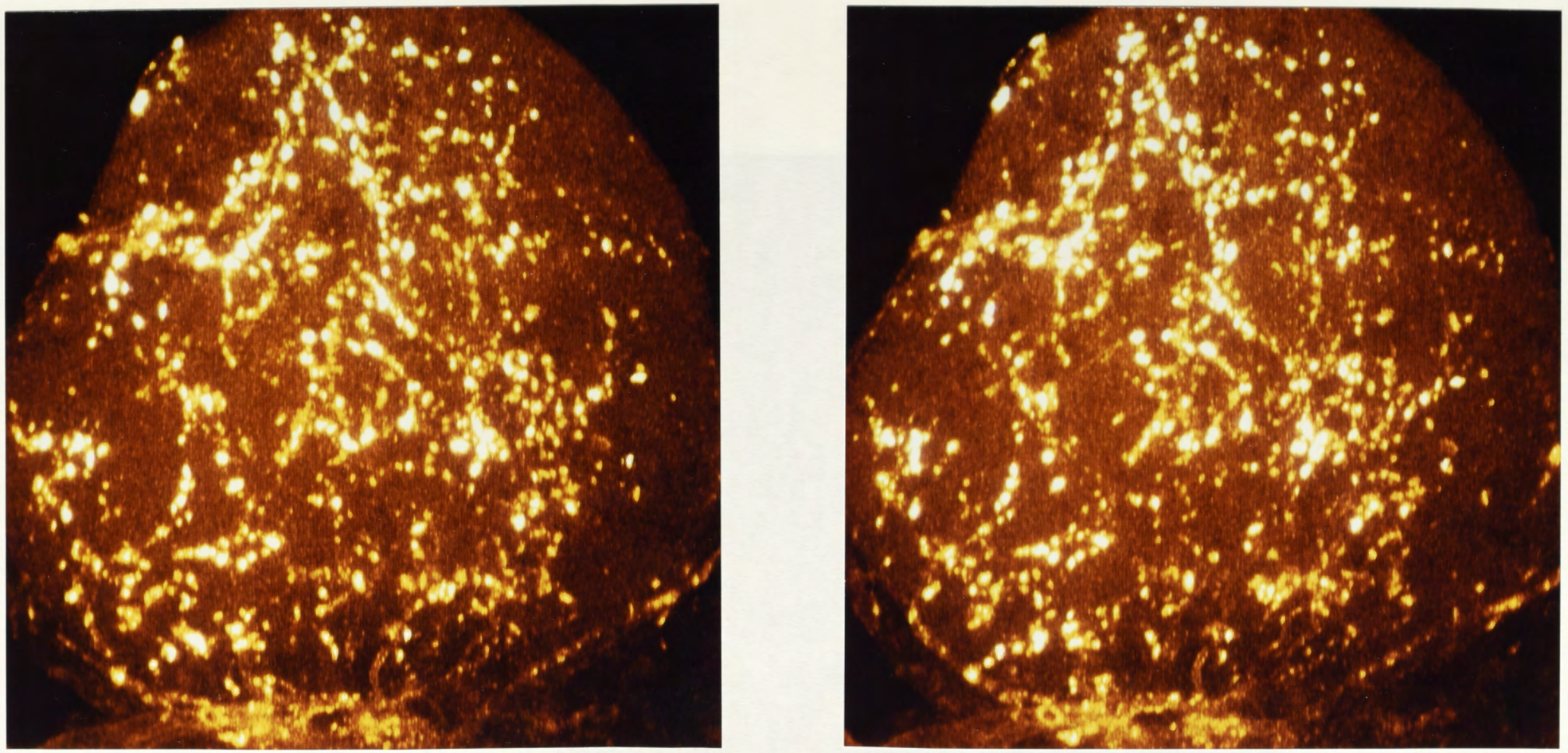


Figure 5.10; Stereopair of the gland shown in Figure 5.9. The images were obtained by rotating the view 6° either side of a centrally positioned vertical axis and creating two new composite pictures. The immunoreactivity can be seen dispersed throughout the interior of the gland, with patches of immunoreactivity surrounding 'empty' spaces corresponding to the CA cells.

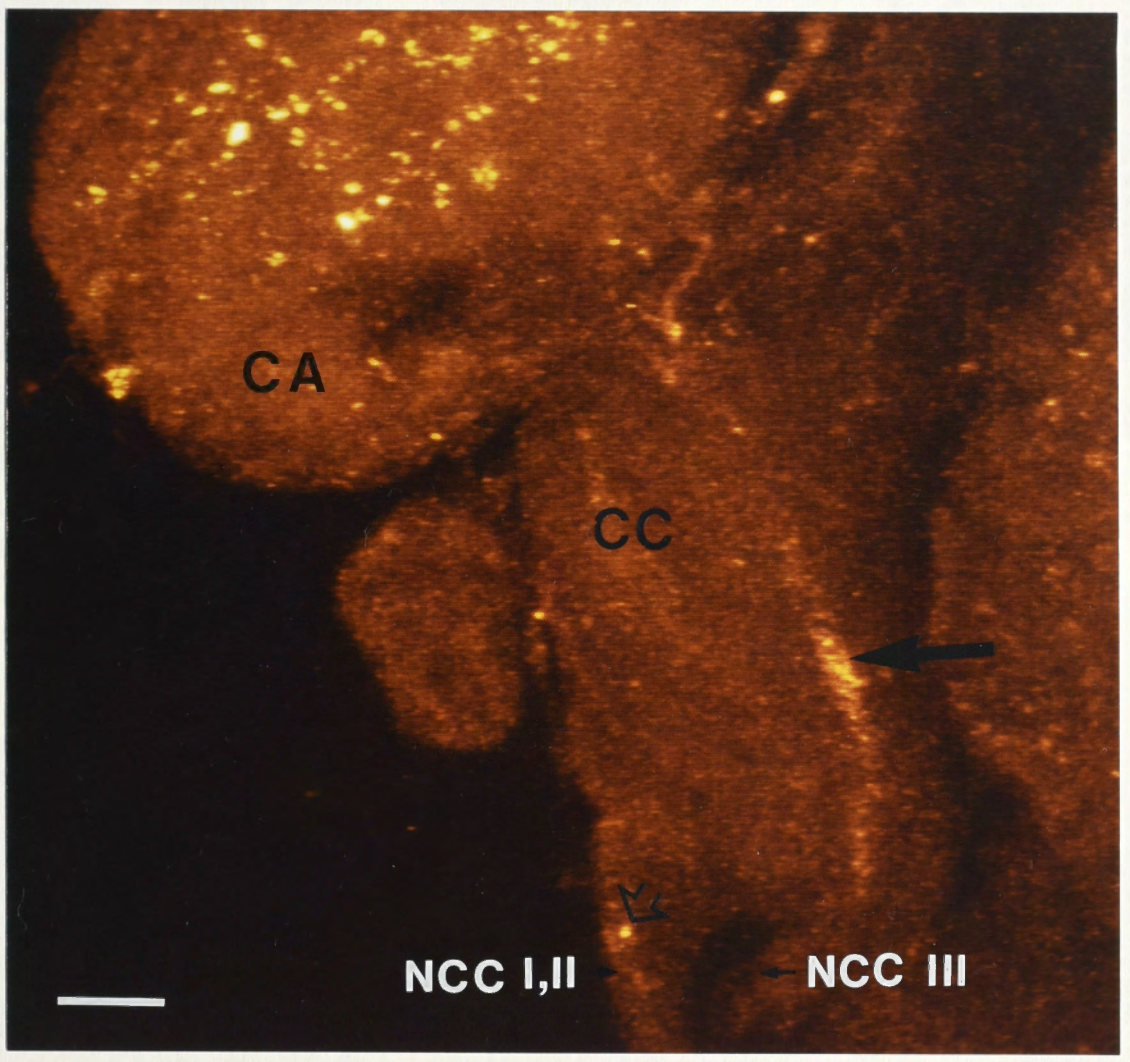


Figure 5.11; Retrocerebral complex from a pharate adult, showing immunoreactive fibres in the CA and lack of immunoreactivity in the CC. The two major nerves to the retrocerebral complex (NCC I/II and NCC III) are indicated, with weak immunoreactivity (open arrow) visible in the NCC I/II. The large arrow indicates autofluorescence associated with trachae lying on the surface of the CC. Scale Bar = 20μm.

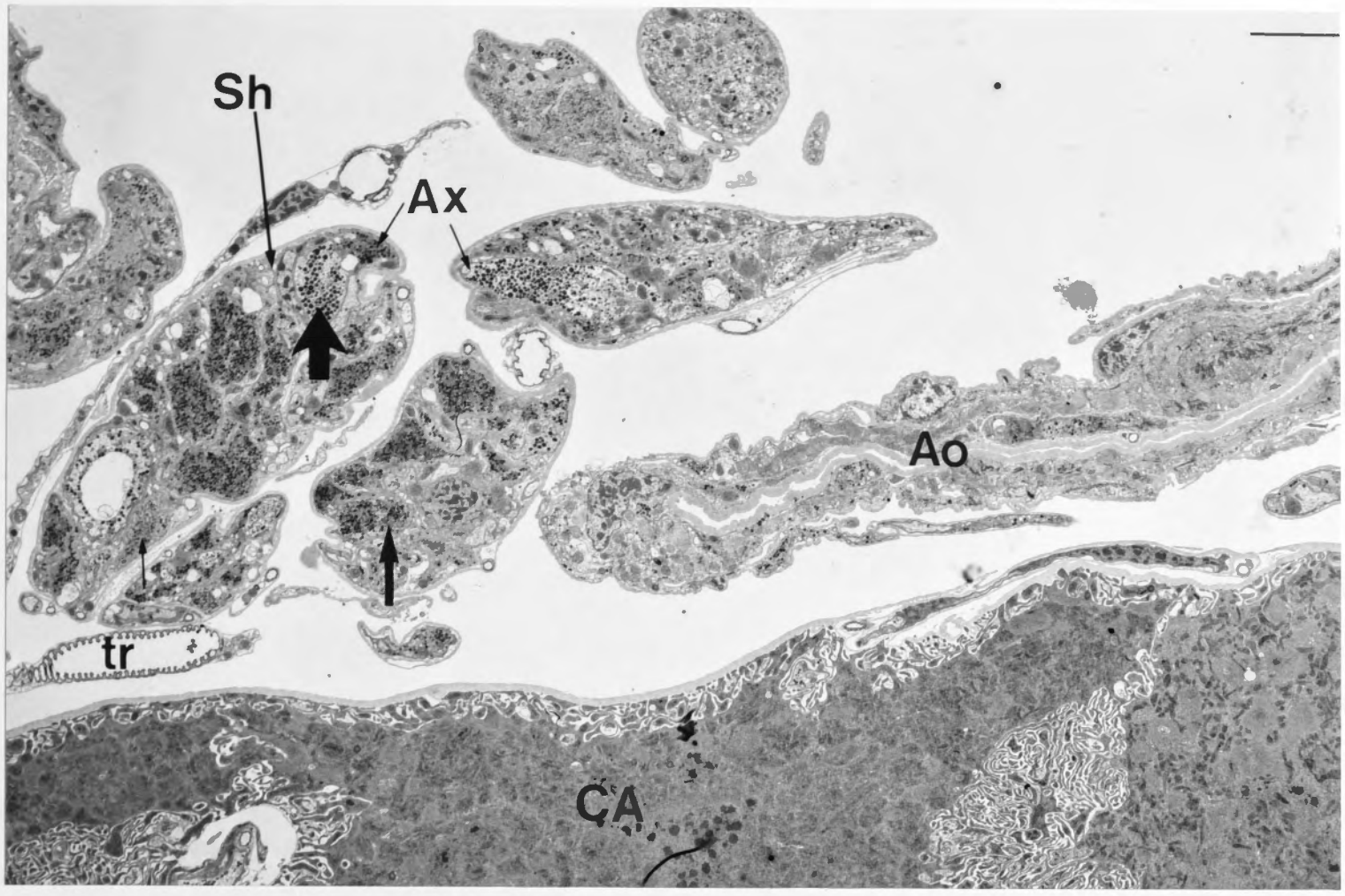


Figure 5.12; Low power transmission electron micrograph (2 250x) showing the edge of the CA and neurosecretory axons in the sheath that surrounds the CA and forms a major neurohemal organ. At least three vesicle types (large, medium and small arrows) are visible. Scale Bar = 4μm.
ABBREVIATIONS; Ao, aorta; CA, corpus allatum; Ax, axons of NSC; Sh, neurohemal sheath; tr, trachea.

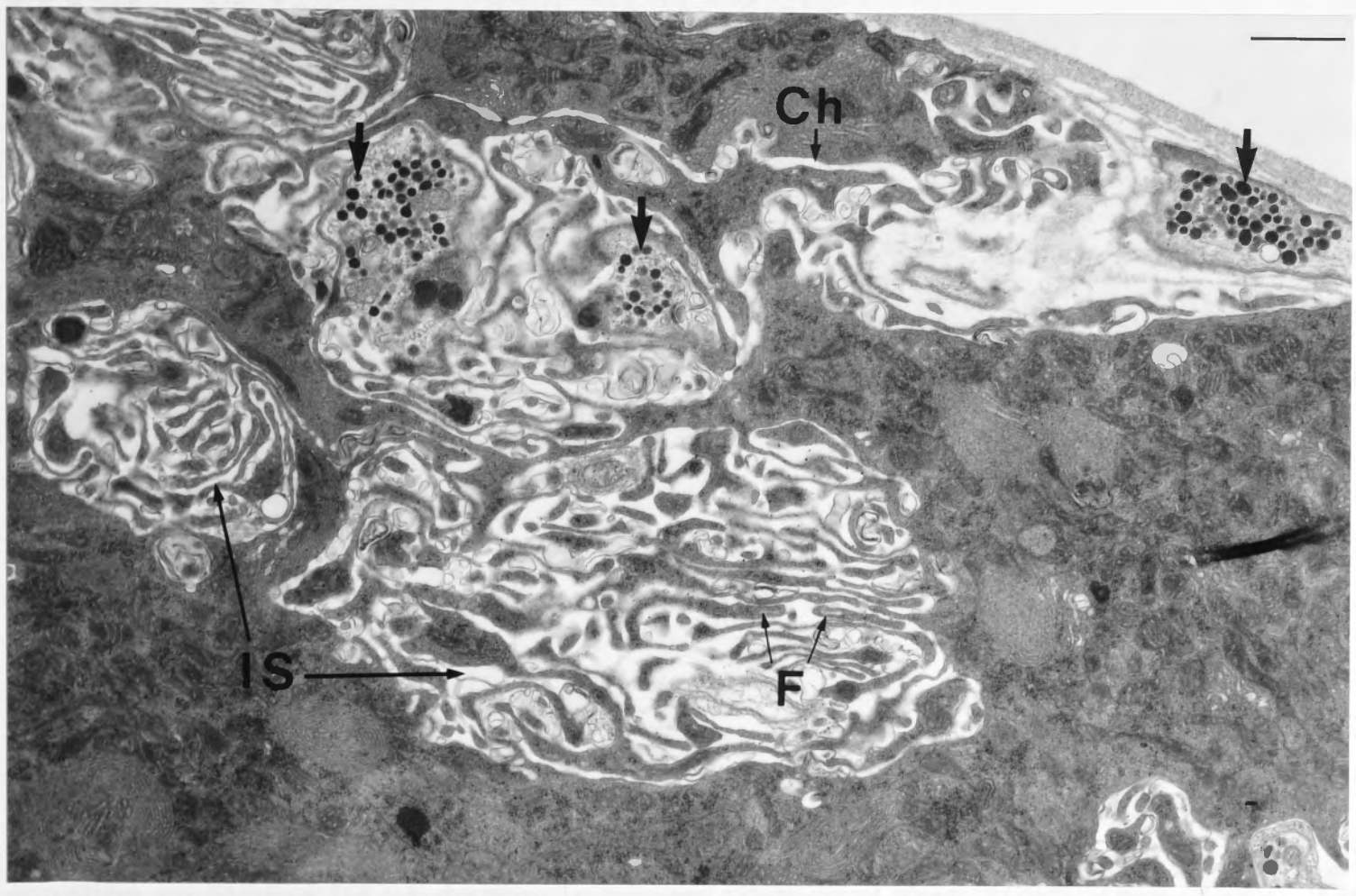


Figure 5.13; Transmission electron micrograph (10 000x) of CA showing several axons with large electron dense vesicles (arrows). The gland contains many spaces between cells that appear to form a complex network of channels (Ch) through the tissue. These intercellular spaces (IS) are packed with lateral membrane folding (F) from surrounding cells. Scale Bar = 1μm.

the CA showing a segment of the aorta and elements of the sheath that envelopes the gland. Nerve axons contained within this sheath were packed with vesicles and several different axon types are apparent based on vesicle morphology. The CA was also richly innervated, with fine axonal processes extending between the gland cells (Figure 5.13 and 5.14). Most nerves contained large, electron dense peptidergic vesicles (Figure 5.13 and 5.14).

Several other structural features which are characteristic of biosynthetically active CA cells were apparent in the *H. armigera* gland. Extensive networks of intercellular spaces were present throughout the tissue (Figures 5.13 and 5.14) with some suggestion that these extended to the edge of the gland (Figure 5.13), providing a possible pathway from the cell to the hemolymph. In addition to axon processes, these spaces were invaded by lateral membrane folding from the surrounding cells (Figures 5.13 and 5.14). The cells contained large numbers of mitochondria (Figure 5.15) and densely packed layers of smooth endoplasmic reticulum (Figures 5.14 and 5.15).

5.4 DISCUSSION

5.4.1 Mas-AT Immunoreactivity in the Brain

Five areas of the brain/sub-oesophageal ganglion of pharate adult/newly eclosed virgin adult female *H. armigera* contained neurons that were immunoreactive with the Mas-AT antiserum; 6-7 pairs of cells in the midline of the brain, 2 pairs of cells in the lateral region of the tritocerebrum, 4 pairs of cells in the lower region of the pars lateralis (Figure 5.1B), 6 pairs of cells in the dorso-lateral region of the protocerebrum and 3 pairs of cells in the sub-oesophageal ganglion (Figure 5.1A). Since Mas-AT and Hea-AT are identical in structure, it was assumed that the cells immunoreacting with the antiserum were expressing the Hea-AT peptide or a related peptide. However the axonal ramifications of these cells could not be followed, and so those cells which innervate the CA and possibly regulate JH synthesis could not be identified. The inability to trace individual axonal projections was probably a function of the quality of the antiserum.

The immediate question that is raised from these observations is which of the immunoreactive cells are controlling JH synthesis in adult female *H. armigera*. There have been few, if any, reports of NSC from the tritocerebrum or sub-oesophageal ganglion regulating JH production in insects (Tobe and Stay, 1985; Khan, 1988). Also, the tritocerebrum innervates the CA via the NCC III and the sub-oesophageal ganglion innervates the CA via the NCA II (Tobe and Stay, 1985) whereas Hea-AT immunoreactivity was only observed in the NCC I/II of *H. armigera* (Figure 5.11). These observations suggest that the immunoreactive cells observed in the tritocerebrum and sub-

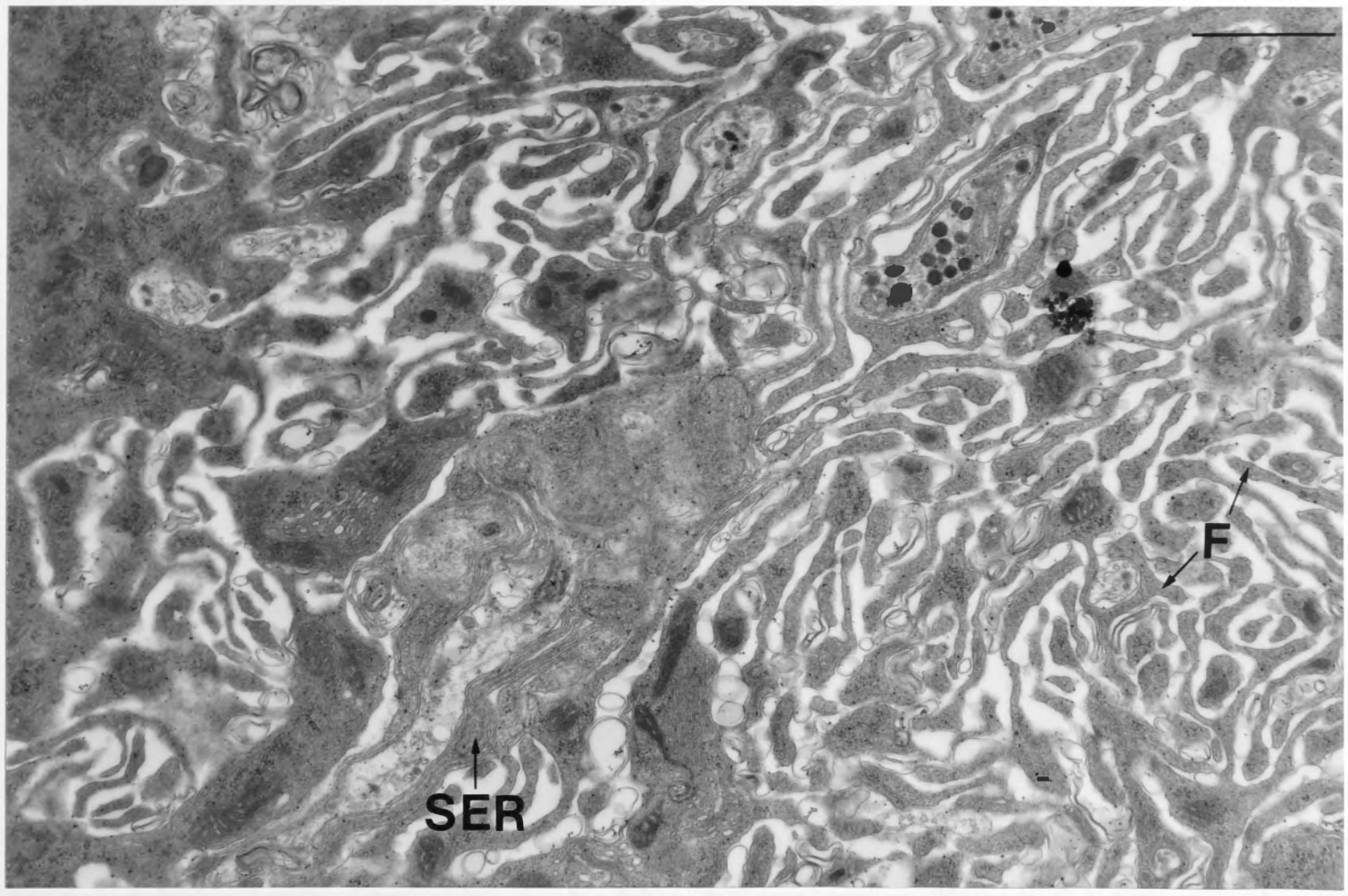


Figure 5.14; Region of CA (20 000x) showing an intercellular space with extensive lateral membrane folding (F). Stacks of smooth endoplasmic reticulum (SER) are clearly visible in the cytoplasm of CA cells. Scale Bar = 1μm.

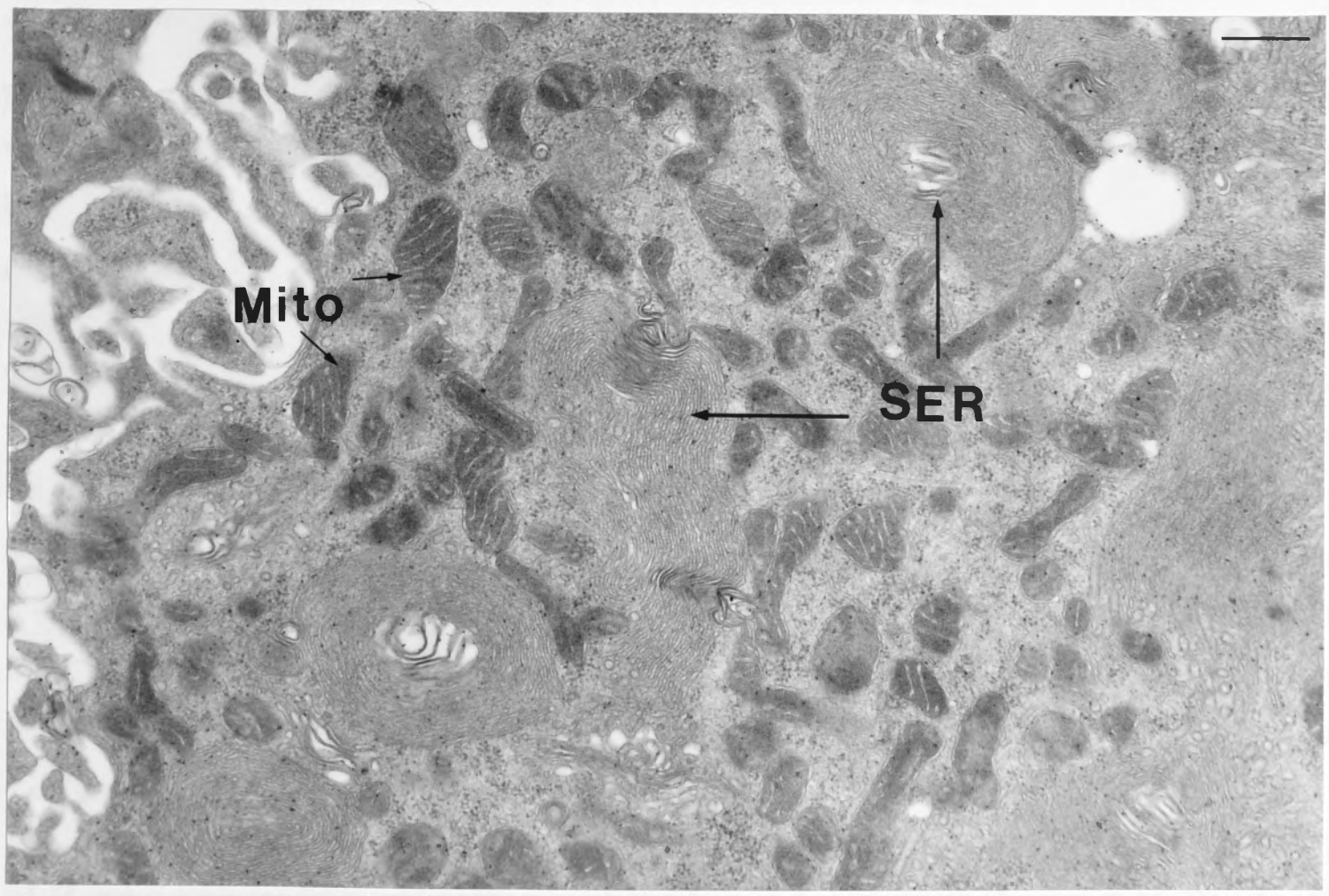


Figure 5.15; Segment of *H. armigera* CA cell (16 000x) showing stacks of smooth endoplasmic reticulum (SER) organized into concentric whorls, and large numbers of mitochondria (Mito) characteristic of biosynthetically active insect CA. Scale Bar = 0.5μm.

oesophageal ganglion are most likely not involved in regulation of JH production in adult female *H. armigera.*

Khan (1988) argued that NSC of the superior pars lateralis play a crucial role in both the stimulation and inhibition of JH synthesis from the CA of insects, whereas the role of MNSC is unclear (Khan, 1988). This generalization is certainly true in virgin adult female *D. punctata* which is by far the best characterized system in regards to the source, axonal pathway and site of release of neuropeptides controlling JH synthesis (section 5.1). The immunoreactive cells in the midline (Figure 5.1B) and the lateral regions of the protocerebrum (Figure 5.1A and B) of the *H. armigera* brain could project axons to the CA via the NCC I/II, which itself is immunoreactive to the Mas-AT antiserum (Figure 5.11). Therefore any of these regions could regulate JH production in adult female *H. armigera*. However in light of the *D. punctata* immunohistochemistry data and Khan's observations (1988), it is most likely that the 6 pairs of lateral cells of the superior protocerebrum immunoreactive to the Mas-AT antiserum (Figure 5.1A and 5.2) are the site of Hea-AT synthesis involved in the stimulation of JH production in adult female *H. armigera.*

Since the only known biological function of the Mas-AT/Hea-AT peptide is the stimulation of JH synthesis by the CA of adult female lepidopterans (Kataoka *et al.*, 1989; Unni *et al.*, 1991; Chapter 3), it might be expected that this peptide would be produced in a limited number of cells within a specific area of the brain. Such is the case with the *M. sexta* prothoracicotropic hormone which is synthesized in only two pairs of LNSC, that project axons to the CA where the peptide is released (O'Brien *et al.*, 1988), and the *M. sexta* eclosion hormone which is synthesized in 5 group Ia cells of the pars intercerebralis whose axons project to the CA where the neuropeptide is released into the hemolymph (Copenhaver and Truman, 1986a). A very different situation is seen with Dip-ASA1 immunoreactivity in the adult female *D. punctata* central nervous system. This immunoreactivity included cells in the lateral region of the protocerebrum which innverate the CA via the NCC II, tritocerebral NSC which innervate the antennal pulsatile muscle via the NCC III, MNSC apparently involved in interneuronal communication within the brain, as well as many other locations (Stay *et al.*, 1992). These findings suggest that the family of *D. punctata* allatostatins (Figure 1.5) may have many functions in adult female cockroaches (Stay *et al.*, 1992).

As discussed above, many cells within the brain and sub-oesophageal ganglion of *H. armigera* are immunoreactive with the Mas-AT antiserum (Figure 5.1). Zitnan *et al.* (1993) have similarily reported many groups of Mas-AT immunoreactive cells in the brain of three other lepidopterans, *Manduca*, *Lymantria* and *Galleria*, as well as *D. melanogaster*. Exact locations of Mas-AT immunoreactivity in the three lepidopteran species were not given and so comparisons to the labelling pattern seen in *H. armigera*

cannot be made. This widespread Mas-AT immunoreactivity suggests that the Mas-AT/Hea-AT peptide has a number of roles in adult female lepidopterans as well as insects from other orders and/or it belongs to a family of peptides within a species that share a common C-terminus, such as the *D. punctata* allatostatins (Figure 1.5).

High pressure liquid chromatography separation of brain extracts prepared from adult *M. sexta*, followed by enzyme-linked immunosorbent assays on the fractions collected with a Mas-AT antibody showed that no peptides related to Mas-AT were present in *M. sexta* (Veenstra and Hagedorn, 1993). This result suggests that the multiple Mas-AT immunoreactive cells in the brain of *M. sexta* (Zitnan *et al.*, 1993) may reflect multiple functions of this peptide in the moth. In *H. armigera* brains, neuropile immunoreactivity within the sub-oesophageal ganglion (Figure 5.7), tritocerebrum (Figure 5.6) and protocerebrum (Figures 5.3-5.4) indicate that Hea-AT may be acting as a neurotransmitter, involved in interneuronal communication within the brain and sub-oesophageal ganglion.

Problems with cross-reactivity of individual antisera with other proteins/peptides is well recognized (Veenstra, 1988; Nässel, 1993). For example 4 neurons in the brain of adult female *L. decemlineata* are recognized by antisera generated against FRMFamide, bovine pancreatic polypeptide and gastrin. However the immunoreactivity seen in these cells is not due to their expressing homologs of each of these peptides but probably is a result of the three antisera recognizing the same motif (RFamide) of a single peptide (Veenstra and Schooveneld, 1984; Veenstra *et al.*, 1984 and 1985). It is therefore possible that the Mas-AT antiserum is immunoreactive with cells in the brain/retrocerebral complex of *H. armigera* that are expressing protein/peptide that is completely unrelated to the Hea-AT peptide. This possibility would best be addressed in *H. armigera* using *in situ* hybridization experiments which allow specific cells which are expressing a target gene to be detected *in situ*. Preliminary experiments using the *Dra I/Cla I* fragment of the Hea-AT genomic phage clone (section 4.3.4) and the Hea-AT cDNA clone (Figure 4.6) as probes to whole mount preparations of adult female *H. armigera* brains, following the protocol of Tautz and Pfeiffle (1989), have been carried out, but problems were encountered with probe penetration in these preparations. Tissue sectioning will most likely circumvent this problem, but this is yet to be completed. Ideally, *in situ* hybridization can be used to identify NSC in the *H. armigera* brain/sub-oesophageal ganglion synthesizing the Hea-AT mRNA after which the pattern of hybridization could be compared to the Mas-AT immunoreactivity data (Figure 5.1). If NSC are detected by Mas-AT immunoreactivity but not by *in situ* hybridization then considerable doubt would be cast over the specificity of the Mas-AT polyclonal antiserum. If the pattern of *in situ* hybridization and immunoreactivity are the same then it is likely that the Hea-AT peptide has additional roles in adult female lepidopterans.

5.4.2 Mas-AT Immunoreactivity in the Retrocerebral Complex

Immunoreactivity to the Hea-AT peptide can be seen throughout the CA of adult female *H. armigera* (Figures 5.8-5.10) indicating that this peptide is released from NSC axons within the CA. A similar result has been reported with Dip-ASA1 immunoreactivity in the adult female *D. punctata* CA (Stay *et al.*, 1992). The situation may be different in adult female *L. migratoria* where the accumulation of allatotropic material within the CC suggests it may be the release site of this peptide(s) (Gadot *et al.*, 1987a). If Mas-AT is delivered to the CA of *M. sexta* as seen in *H. armigera*, then the NSC identified by Copenhaver and Truman (1986b) (see Figure 1.1) are probably not involved in the allatotropic regulation of CA activity, as axons of these NSC did not terminate within the CA itself.

Hea-AT appears to reach the CA via the NCC I/II (Figure 5.11). This result is consistent with experiments on adult female *M. sexta* where NCC I/II denervation prior to eclosion caused an approximate 10 fold decrease in the number of eggs matured (Sasaki and Riddiford, 1984; Ishizaka *et al.*, 1987).

Variations were noted in the intensity of Mas-AT immunoreactivity within the CA of newly eclosed female *H. armigera* (section 5.3.2). This may simply be a result of individual variation and have no biological significance. However it could be related to the timing of Hea-AT release from axon terminals. Release of the allatotropin may occur in bursts and could be correlated with the timing of CA sensitivity to the peptide (section 3.4.3). The exact age of the moths when they were sacrificed was not recorded, and so attempts to relate the time of eclosion with the quantity of immunoreactive material cannot be made. The circadian control of neurosecretion has previously been observed in a number of insect species (Beck, 1980), including the release of prothoracicotropic hormone from the CA of *Samia cynthia ricini* (Fujishita and Ishizaki, 1981; Mizoguchi and Ishizaki, 1984) and serotonin release from the retrocerebral complex of cockroaches (Jagota and Habibulla, 1992).

5.4.3 *H. armigera* CA Ultrastructure

Preliminary ultrastructural studies of the NSC axons within the CA suggest that these axons ramify both in the sheath of adult female *H. armigera* CA and within the CA itself (Figures 5.12 and 5.13 respectively). A similar finding has been made in larval *M. sexta* CA by Sedlak (1981), who hypothesized that NSC axons within the sheath are involved in the neurohemal function of the CA and those within the CA itself are involved in the regulation of gland function. Immunohistochemical studies with the prothoracicotropic hormone of *M. sexta* has shown that it is indeed released from neurosecretory terminals in the sheath of the CA (O'Brien *et al.*, 1988). Therefore it

seems probable that the axons which ramify in the sheath of the *H. armigera* CA (Figure 5.12) also release their contents into the hemolymph. Neurosecretory axons that project into the CA of *H. armigera* have been shown to contain the Hea-AT immunoreactive material (Figure 5.8-5.10) and therefore are likely to be involved in regulation of the glandular function of the CA.

Numerous and large intercellular spaces can be seen within the adult female *H. armigera* CA (Figure 5.13 and 5.14). In other insects intercellular spaces of the CA are narrow and moniliform in inactive glands, but in active glands they become considerably more prominent (Waku and Gilbert, 1964; Haget *et al.*, 1981; Fukuda *et al.*, 1966). These intercellular spaces are probably important for the supply of energy and precursors for JH production, as well as for the release of JH into the hemolymph (Cassier *et al.*, 1990). Neurosecretory axons within the CA appear to be associated with these intercellular spaces (Figure 5.13 and 5.14), indicating that neurosecretory substances, such as Hea-AT, are probably released into these spaces.

In the CA of adult female *H. armigera* mitochondria are found at a high density (Figure 5.13 and 5.15). In inactive CA of other species the mitochondria are scarce and globular or rod shaped, whereas in active glands the density of mitochondria increases dramatically and they are often dumbbell, cup or ring shaped (Waku and Gilbert, 1964; Fukuda *et al.*, 1966; Melnikova and Panov, 1975; Sedlak *et al.*, 1983). Mitochondria are needed to fulfil the high energy demands of active CA, which require ATP and NAD+ for the JH biosynthetic pathway (Figure 1.3).

Adult female *H. armigera* CA contain large quantities of concentric smooth endoplasmic reticulum (Figure 5.14 and 5.15) which have been considered an index for the glandular activity of the CA (Cassier, 1990). Whorls of concentric smooth endoplasmic reticulum have been noted in active CA from many other insect species (Deleurance and Charpin, 1978; Yin and Chippendale, 1979b; Sedlak *et al.*, 1983). Two enzymes of the JH biosynthetic pathway, HMG CoA reductase and 10,11 epoxidase, are localized within the endoplasmic reticulum and it has also been suggested that this compartment may play a role in the movement of JH out of the cell (Tobe and Stay, 1985).

Also of significance is the distinct difference in the CA volume which can be seen between pharate adult and adult *H. armigera* (Figures 5.8 and 5.9, respectively). This observation is consistent with the idea that during the transition from pharate adult to eclosed adult, the capacity of the CA to synthesize JH is being established through an increase in cell size and/or cell volume. Such correlations with CA activity and volume have previously been noted in *Nauphoeta cinerea* (Lanzrein *et al.*, 1978), *D. punctata* (Johnson *et al.*, 1985), *Blattella germanica* (Chiang *et al.*, 1991) and *D. melanogaster* (Dai and Gilbert, 1991).

5.5 SUMMARY

Numerous cells within the brain and sub-oesophageal ganglion of *H. armigera* were immunoreactive with the Mas-AT antiserum, which has also been observed in other lepidopterans as well as larval and adult *D. melanogaster* (Zitnan *et al.*, 1993). Present evidence indicates that no other peptides related to Hea-AT exist in *H. armigera*, and that this peptide may have a number of functions within the moth. However, the possibility exists that not all of the cells immunoreactive with the antiserum are producing Hea-AT because it cannot be excluded that the antibody was binding to unrelated peptides.

Although the cells of the brain which synthesize Hea-AT and innervate the CA could not be unequivocally identified, it has been hypothesized that one or more of 6 pairs of NSC in the superior lateral protocerebrum are the source of the Hea-AT peptide which stimulates JH production from the CA of adult female *H. armigera*. Evidence suggests that the axons from these cells reach the CA via the NCC I/II and release the Hea-AT peptide within the CA in close proximity to the JH producing cells.

Ultrastructural analysis of the adult female *H. armigera* CA shows that NSC axons ramify in the sheath of the CA and within the CA itself. Large quantities of intracellular spaces, mitochondria and smooth endoplasmic reticulum, which have been observed in active CA of other insect species, were noted in adult female *H. armigera* CA.

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6

6.1 INTRODUCTION

The research presented in this thesis has been directed towards an understanding of the neuroendocrine regulation of juvenile hormone production in virgin adult female *Helicoverpa armigera* (Lepidoptera:Noctuidae). The major emphasis has been on the identification of an *H. armigera* allatotropic peptide through the molecular biological characterization of the gene and deduced preprohormone that encode it.

During the course of this work, a number of factors involved in the production of mature eggs in female *H. armigera* have been examined. These are summarized in Figure 6.1. It has been determined that an mRNA encoding an allatotropic peptide (Hea-AT) is synthesized in the brain of *H. armigera* (section 3.4.1), possibly in the lateral neurosecretory cells (LNSC) of the superior protocerebrum (section 5.4.1). The Hea-AT peptide is putatively transcribed from a gene that consists of at least two exons (section 4.4.2) and produces a mRNA that encodes a preprohormone that is at least 54 amino acids long. This partial Hea-AT preprohormone sequence contains; a single copy of the Hea-AT peptide, a potential hydrophobic signal sequence, endopeptidase recognition sites and a glycine α-amidation substrate. The predicted Hea-AT peptide is identical in sequence to the Mas-AT peptide (section 4.4.3). Hea-AT appears to be transported to the CA via the NCC I/II where it is released from axon terminals uniformly distributed throughout the CA (section 5.4.2). During pharate adult development the CA becomes competent to synthesize JH, a process that involves activation or *de novo* production of the JH biosynthetic pathway enzymes (section 2.4.3) and an increase in gland volume (section 5.4.2). JH production from the CA is initiated around the time of adult eclosion (section 2.4.3), and there was some indication that subsequent synthesis of JH may be under circadian control (section 3.4.3). JH III is the major product of unstimulated CA *in vitro* (section 2.4.2). Unexpectedly, JH diol is also a product of these glands (Figure 2.5A), and its release is greatly increased when CA are incubated in the presence of FA or allatotropin (Figures 2.5B, 3.3B and 3.3C). The *in vivo* biological significance of JH diol is unknown. JH degrading esterase activity is found in association with the CA and/or its surrounding tissue (section 2.4.2). Finally it has been unequivocally shown that without JH, oocyte maturation does not occur in adult female *H. armigera.*

In the following sections the major findings of my work are dealt with in more detail. The neuroendocrine regulation of JH production is discussed, with particular emphasis on an experimental strategy for the complete characterization of the Hea-AT

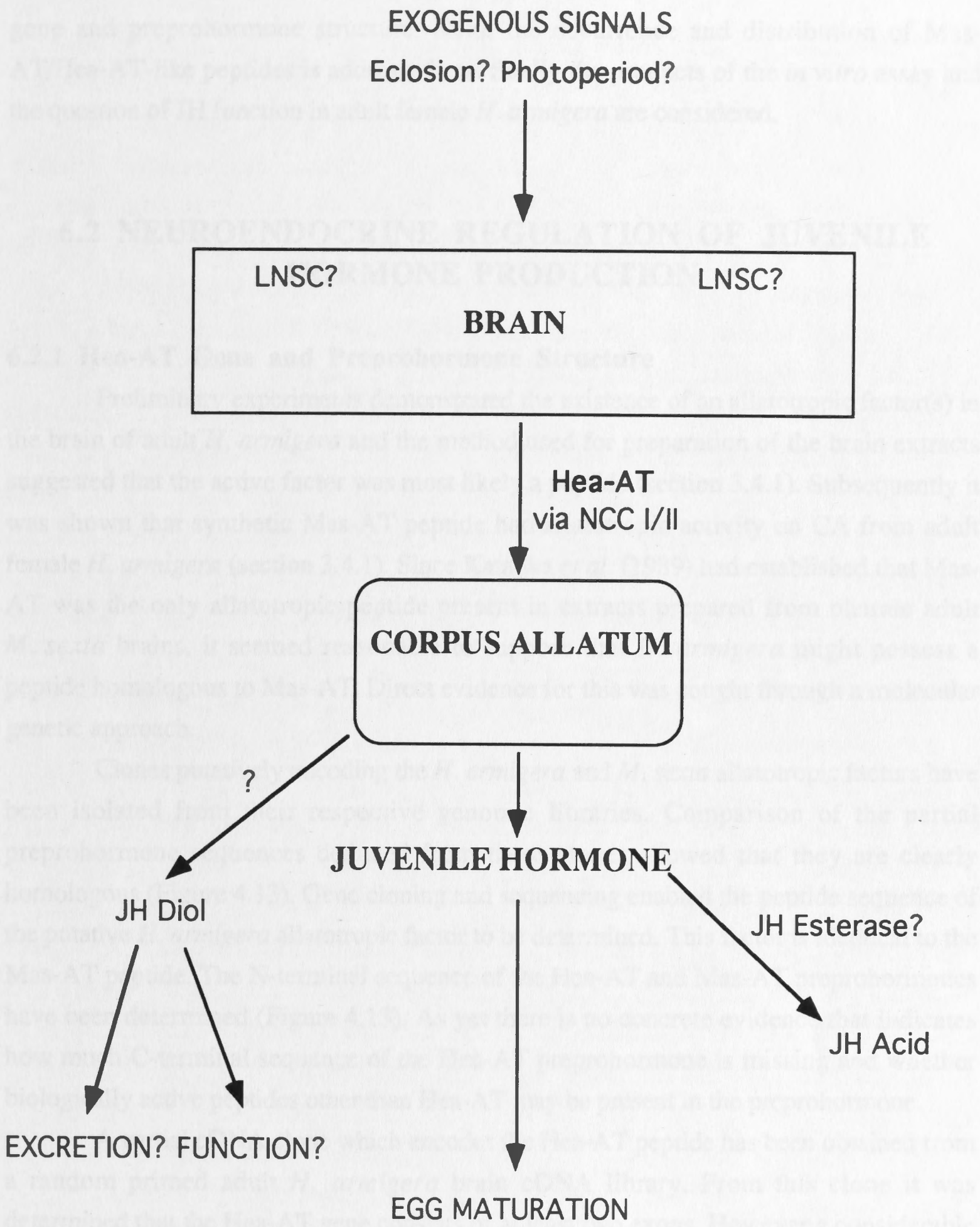


Figure 6.1; Aspects of the neuroendocrine regulation of egg maturation in virgin adult female *H. armigera* examined in this thesis. These studies have implicated several components involved in the neuroendocrine regulation of JH production and have identified unexpected enzymatic activities associated with the CA. The identities and significance of these factors, which are indicated by question marks, remain unknown.

gene and preprohormone structure. Next, the occurrence and distribution of Mas-AT/Hea-AT-like peptides is addressed and finally the products of the *in vitro* assay and the question of JH function in adult female *H. armigera* are considered.

6.2 NEUROENDOCRINE REGULATION OF JUVENILE HORMONE PRODUCTION

6.2.1 Hea-AT Gene and Preprohormone Structure

Preliminary experiments demonstrated the existence of an allatotropic factor(s) in the brain of adult *H. armigera* and the method used for preparation of the brain extracts suggested that the active factor was most likely a peptide (section 3.4.1). Subsequently it was shown that synthetic Mas-AT peptide had allatotropic activity on CA from adult female *H. armigera* (section 3.4.1). Since Kataoka *et al.* (1989) had established that Mas-AT was the only allatotropic peptide present in extracts prepared from pharate adult *M. sexta* brains, it seemed reasonable to suppose that *H. armigera* might possess a peptide homologous to Mas-AT. Direct evidence for this was sought through a molecular genetic approach.

Clones putatively encoding the *H. armigera* and *M. sexta* allatotropic factors have been isolated from their respective genomic libraries. Comparison of the partial preprohormone sequences deduced from these clones showed that they are clearly homologous (Figure 4.13). Gene cloning and sequencing enabled the peptide sequence of the putative *H. armigera* allatotropic factor to be determined. This factor is identical to the Mas-AT peptide. The N-terminal sequence of the Hea-AT and Mas-AT preprohormones have been determined (Figure 4.13). As yet there is no concrete evidence that indicates how much C-terminal sequence of the Hea-AT preprohormone is missing and whether biologically active peptides other than Hea-AT may be present in the preprohormone.

A partial cDNA clone which encodes the Hea-AT peptide has been obtained from a random primed adult *H. armigera* brain cDNA library. From this clone it was determined that the Hea-AT gene consists of at least two exons. However a considerable amount of work remains to be done before the complete Hea-AT gene and preprohormone structure is known. The pathway to reach these goals essentially involves two processes: the characterization of the complete Hea-AT message and gene sequence. Methods to achieve these objectives are outlined below.

Isolation of a full length cDNA, or a series of overlapping partial cDNA clones would allow the unambiguous determination of the peptide preprohormone structure. This would best be approached by screening a library prepared from a mixture of oligo-dT primed and random hexamer primed cDNA pools. Oligo-dT primed cDNA will permit

the identification of the 3' end of the mRNA, starting at the site of polyadenylation. Since full length cDNA clones are rarely obtained from oligo-dT primed libraries, the inclusion of random hexamer primed cDNAs facilitates the identification of the 5' end of the mRNA. However an alternative to this approach was taken because previous attempts to produce oligo-dT primed cDNA libraries in our laboratory had not been successful (P. East, personal communication).

Preliminary northern blot analysis using the *Dra I/Cla I* fragment of the Hea-AT genomic clone (section 4.3.4) as a hybridization probe suggested that the Hea-AT mRNA was less than 500bp in length. To increase the probability of obtaining Hea-AT cDNA clones it was decided to prime the first strand cDNA synthesis with a high ratio of random hexamers relative to mRNA. Sequence analysis of the clone obtained from this cDNA library showed that this approach, although successful in yielding a partial Hea-AT cDNA, has a major drawback. The clone obtained was almost certainly a hybrid of two or more independent cDNA fragments (section 4.4.2). In hindsight, this might have been predicted, as this method of cDNA synthesis was designed to produce large numbers of small cDNA fragments.

In principle, now that partial cDNA sequence is available, complete characterization of the mRNA could be achieved through available methods based on PCR (Polymerase Chain Reaction). These methods are generically known as RACE (Rapid Amplification of cDNA Ends) PCR and a number of variant procedures have been developed to permit identification of the 3' and 5' ends of mRNAs where internal sequences are known (Ohara *et al.*, 1989; Frohman, 1990; Jain *et al.*, 1992; Trout *et al.*, 1992). These methods are technically demanding, requiring large numbers of sequential enzymatic reactions. In the latter stages of my research, several attempts were made to apply both 5' and 3' RACE-PCR methods for characterization of the Hea-AT mRNA, but without success. It would seem then, that the best option for obtaining complete cDNA sequence information is the construction of a new adult brain cDNA library from oligo-dT primed and random hexamer primed cDNA pools.

Once the complete mRNA sequence of the Hea-AT gene is determined, two major aims of my research can be completed. Firstly, the cloned mRNA can be used as a probe to characterize the structure and sequence of the Hea-AT gene with standard molecular biology techniques already presented in Chapter 4. Secondly the complete open reading frame of the Hea-AT mRNA could be determined and analysed for the presence of other possible neuropeptides, both related and/or unrelated to Hea-AT.

6.2.2 Mas-AT/Hea-AT Peptide Family

When first characterized, the only known function of the Mas-AT peptide was its ability to stimulate JH synthesis from the CA of adult female lepidopterans. However,

further research suggests that its distribution and roles within the Insecta is far more complex.

Several cells within the brain of *H. armigera* were immunoreactive with the Mas-AT antiserum (Figure 5.1). At least three possible explanations can account for the relatively high numbers of immunoreactive sites; 1) there may be other, related peptides produced from the same or a different prohormone, 2) the Hea-AT peptide may have more than one function and consequently is expressed in a number of cell types, 3) the Mas-AT antiserum is binding to peptides/proteins which are unrelated to Hea-AT. To begin to address these possibilities in *H. armigera,* two types of experiments previously discussed could be used. Firstly the complete Hea-AT preprohormone sequence needs to be characterized (section 6.4) so that the number of Hea-AT related peptides can be determined. Secondly, *in situ* hybridization experiments (section 5.4.1) using a Hea-AT cDNA clone as a probe, would establish which cells in the brain are expressing the Hea-AT gene. If *in situ* hybridization detects the same cells of the brain as the Mas-AT antiserum, and the Hea-AT preprohormone only has one Hea-AT related peptide, then the likelihood of the Hea-AT peptide having multiple functions in adult female *H. armigera* is considerably strengthened. On the other hand, if the *in situ* hybridization pattern is restricted to a subset of the Mas-AT immunoreactive cells in the brain, then these cells would probably be controlling JH synthesis. The other immunoreactive cells could be producing Hea-AT related peptides on another preprohormone gene or the antibody may be cross-reacting with an unrelated peptide(s).

Recent studies tend to favour the idea that peptides related to Mas-AT are phylogenetically widespread in insects, are expressed at multiple sites in the central nervous system and possibly are present as a single peptide in many species. The same antiserum that was used in this study has been utilized for an immunohistochemical study of the dipteran, *D. melanogaster*, and three lepidopteran species (Zitnan *et al.*, 1993). For *D. melanogaster*, multiple sites of immunoreactivity were observed in the central nervous system. The results for the lepidopterans were not presented, but multiple immunoreactive cells were reported to be present in the brain of all three species. A different antiserum has been prepared against the Mas-AT peptide, and was used by Veenstra and Hagedorn (1993) to monitor high pressure liquid chromatography fractions of central nervous system extracts from *M. sexta* and the cockroach, *P. americana.* In both species a single immunoreactive fraction was observed, which coeluted with synthetic Mas-AT. Finally, a myotropic peptide isolated from male accessory sex glands of *L. migratoria* (Paemen *et al.*, 1991) appears to be a structural homolog of Mas-AT. This peptide is 13 amino acids long, is amidated at its carboxyl terminus, and is identical in sequence to Mas-AT at 10 out of 13 residues. An antiserum produced against this peptide identified multiple sites in the locust central nervous system (Paemen *et al.*,

1992). Taken together, these results suggest that a peptide related to Mas-AT is present in species representing four different orders; Lepidoptera, Diptera, Orthoptera and Blattodea. The allatotropic activity of Mas-AT appears to be restricted to adult female lepidopterans (Kataoka *et al.*, 1989), but the pattern of Mas-AT immunoreactivity in the *H. armigera* brain is consistent either with additional peptide functions in this species or with antibody cross-reactivity. A repeat of the immunohistochemical study, using the antiserum of Veenstra and Hagedorn (1993) would be most informative, as this serum was reported to be specific for the Mas-AT peptide.

6.3 JUVENILE HORMONE SYNTHESIS AND OOGENESIS

A complete understanding of the neuroendocrine cascade regulating egg production in *H. armigera* will require information on both the neurohormonal factors (such as the Mas-AT/Hea-AT peptide) and their receptors on the CA that control JH synthesis, and on homologs of JHs, and their receptors, that control oocyte maturation. Although allatotropic peptides were the main focus of this thesis, the *in vitro* JH assay used to monitor allatotropin activity has provided useful insights into other aspects of JH production in *H. armigera.*

6.3.1 Activation of the CA in Virgin Adult Female *H. armigera*

Juvenile hormone synthesis is shut down during the final larval instar in holometabolous insects to allow pupation to proceed (section 1.3.1). Following metamorphosis the CA must be reactivated to provide JH for adult reproductive functions (section 1.3.2 and Appendix 1). This process has not been investigated to any significant extent in lepidopterans, but it is possible that the adult specific allatotropin, Mas-AT, is one component involved in gland activation. Juvenile hormone production by the CA of *H. armigera* is initiated around the time of adult eclosion (section 2.4.3, Figure 2.7). This finding is consistent with observations in the closely related species *H. zea,* where JH synthesis begins just prior to eclosion (Satyanarayana *et al.*, 1991).

Two observations suggest that the capacity of the CA to synthesize JH is being established during pharate adulthood. Firstly, a major increase in the ability of CA to convert FA to JH III was observed as the moths went from pharate adults to adults (Figure 2.8). This indicates that during pharate adulthood the biosynthetic capacity of the CA to carry out the two terminal enzymatic steps of the JH biosynthetic pathway are being put in place. Secondly, as female *H. armigera* go from pharate adults to adults, the volume of the CA increases (Figures 5.8 and 5.9). Although not directly examined in this study, this phenomenon is probably at least partly explained by an increase in cell volume

associated with the production of large quantities of mitochondria and smooth endoplasmic reticulum (section 5.4.3) as well as expansion of the spaces between individual cells (section 5.4.3). Such CA volume and ultrastructural changes have previously been observed during the pupal-adult transformation in *D. melanogaster* (Dai and Gilbert, 1991).

6.3.2 Products Released from the *H. armigera* CA *in vitro*

The Lepidoptera are unusual among insects in that they produce multiple JH homologs, where most other species produce only one (section 1.2.2). In adult female lepidopterans, JH II and JH III are generally more abundant than JH I. Chromatographic analysis of products released by the *H. armigera* CA showed JH III to be the major homolog, representing more than 70% of the radiolabelled material identified (Figure 2.5A). This is somewhat different to results obtained for other adult female lepidopterans, where JH II is usually the major homolog released from the CA (Cusson *et al.*, 1990; Unni *et al.*, 1991). It should be noted, however, that the *H. armigera* estimates were taken from moths of only one age. When examined, homolog ratios have been found to vary with age within a species (Ishizaka *et al.*, 1987; Satyanarayana *et al.*, 1991) and this may also be true of *H. armigera*.

The biological significance of the CA producing more than one type of JH is unknown (Cassier, 1990). This problem of homolog function is likely to prove difficult to solve, as there are many inconsistencies between different studies. For example, homolog ratios differ between species, and as noted above, vary with age within a species. In addition, Bhaskaran *et al.* (1986) argued that the difference between their results and those of Granger *et al.* (1982) for the ratio of JH homologs released from prepupal *M. sexta* CA was due to a strain difference. Various hypotheses have been proposed to explain differential rates of JH homolog production. For example, it has been suggested that neuroendocrine factors may play a role in determining the ratios of JH homologs released by lepidopteran CA. However there is no evidence that allatotropins preferentially stimulate synthesis of a particular JH homolog in adult female *M. sexta* (Unni *et al.*, 1991) or *H. amigera* (section 3.3.3), so this explanation cannot be generally applied. An alternative, proposed by Schooley and Baker (1985), is that JH homolog ratios are determined by the availability of specific precursors, such as propionate which is required for the synthesis of higher homologs of JH. Differential precursor supply could occur via the controlled production of propionate within the lepidopteran CA through enzymes such as branched-chain amino acid transaminases which can produce propionate from certain amino acids (e.g., isoleucine) (Brindle *et al.*, 1992), although this hypothesis is yet to be tested experimentally.

Another finding of my studies with adult female *H. armigera* deserves further

investigation. In addition to JH III, significant quantities of JH diol (approximately 20% of the identified product, Figure 2.5A) were released from *H. armigera* CA. The quantity of JH diol relative to JH increased dramatically in experiments with Mas-AT and FA, where total JH production was stimulated (Figures 3.3B and C, and Figure 2.5B, respectively). The reasons for this are unknown, and several possibilities have been discussed (section 2.4.2). Release of JH diol has previously been reported for only one other insect species, the locust *L. migratoria* (Gadot *et al.*, 1986 and 1987b), in which allatotropic or FA stimulation of JH synthesis resulted in the preferential release of JH III over JH III diol (Gadot *et al.*, 1986). The release of the JH diols raises the theoretical possibility that this compound, which is normally considered to be a product of JH degradation, may in some instances have a functional role.

Most of what is known about the identities and relative quantities of compounds released from insect CA derives from *in vitro* data. It has been noted that the relative quantities of different JH homologs measured in the RCA may be poor indicators of *in vivo* values in lepidopterans (Schooley and Baker, 1985; Cusson *et al.*, 1990). Clearly it is the levels of circulating hormone *in vivo* that are biologically relevant, and it is desirable that this information be obtained for *H. armigera* in future studies. Nevertheless, the *in vitro* assay has been useful for identifying compounds released by the *H. armigera* CA and the information will serve as a valuable guide for the determination of hemolymph hormone titres. Since measurement of hemolymph JH. usually relies on direct chemical determinations involving use of mass spectrometry, the recovery of JH diol from the RCA suggests that ions diagnostic for these molecules should be included in any future analysis of *H. armigera* CA products.

6.3.3 JH and Oogenesis

It will not be realistic to commence detailed studies of JH function in *H. armigera* until hemolymph hormone titres are known. In addition, it will probably not be possible to determine the functions of individual JH homologs until specific cellular receptors have been identified. The roles of JH in egg maturation vary considerably between insect species (Bownes, 1986). Of those lepidopteran species that require JH for egg maturation (Table 1.1, group II), only two have been investigated in any detail. One of these, *H. zea*, is a close relative of *H. armigera* and it is likely that the roles of JH in oocyte production will be the same for these two species. In *H. zea*, JH is required for the initiation of vitellogenin synthesis by the fat body and uptake of circulating vitellogenin by the developing oocyte (Satyanarayana *et al.*, 1992). An essential role for JH in egg maturation within *H. armigera* has been established, since removal of the CA via decapitation abolished ooctye production, and subsequent injection of JH III or the JH analog, methoprene, restored vitellogenesis to the levels of, or above, undecapitated

controls (section 2.4.1, Table 2.1). Identification of the cellular targets of JH action is a prerequisite for identification of hormone receptors, which remains an outstanding problem in insect JH research.

6.4 CONCLUDING REMARKS

This study provides the first information on a molecular basis for the neuroendocrine regulation of female reproduction in *H. armigera*. Neuroendocrine systems, such as the regulation of JH synthesis during egg maturation, have been proposed as targets for the rational design of novel insect pest control procedures, but as yet, the means by which such systems can be attacked do not exist (Keeley and Hayes, 1987; Evans *et al.*, 1989; Menn and Borkovec, 1989; Kelly *et al.*, 1990; Masler *et al.*, 1993). Knowledge of the molecular mechanisms that operate in controlling JH synthesis is an essential first step towards identifying vulnerable targets.

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APPENDICES

APPENDIX 1

Roles of JH in the insect life cycle, in addition to metamorphosis (section 1.1.3.1) and oocyte maturation (section 1.1.3.2).

A) Insect polymorphism/phase

i) Determination of solitary and gregarious forms, some examples;

Leucania separata	(Kumaran, 1990)
Schistocerca gregaria	(Injeyan and Tobe, 1981)
Spodoptera litura	(Kumaran, 1990).

ii) Diapause, some examples;

Diatraea grandiosella	(Yin and Chippendale, 1979a)
Laspeyresia pomonella	(Friedländer, 1982)
Leptinotarsa decemlineata	(deWilde and de Boer, 1969)
Locusta migratoria	(Poras *et al.*, 1983)
Musca autumnalis	(Burks *et al.*, 1992)
Monema flavescens	(Takeda, 1978).

iii) Colouration, an example;

Schistocerca gregaria	(Pener, 1965).

iv) Migratory behaviour/wing dimorphism (see Pener, 1985), some examples;

Gryllus rubens	(Zera and Tobe, 1990)
Ips confusus	(Unnithan and Nair, 1977)
Locusta migratoria	(Kumaran, 1990)
Oncopeltus fasciatus	(Rankin and Riddiford, 1978)
Schistocerca gregaria	(Kumaran, 1990).

v) Caste determination (see Hardie and Lees, 1985), some examples;

Apis mellifera carnica	(Rachinsky and Hartfelder, 1991)
Bombus terrestris	(Röseler and Röseler, 1978)
Myrmica rubra	(Brian, 1974)
Polistes gallicus	(Röseler *et al.*, 1980)
Zootermopsis angusticollis	(Greenberg and Tobe, 1985).

B) Reproduction (other than egg maturation), see; Engelmann (1970), Koeppe *et al.* (1985) and Raabe (1989).

i) Female left colleterial gland development and function, some examples;

Blattella germanica	(Burns *et al.*, 1991)
Nymphalis antiopa	(Herman and Bennett, 1975)
Periplaneta americana	(Weaver, 1981).

ii) Male accessory sex gland and/or tubular ejaculatory duct development and function, some examples;

Blattella germanica	(Piulachs *et al.*, 1992)
Drosophila sp.	(Yamamoto *et al.*, 1988)
Nymphalis antiopa	(Herman and Bennett, 1975)
Schistocerca gregaria	(Avruch and Tobe, 1978).

iii) Spermatogenesis, some examples;

Laspeyresia pomonella	(Friedländer, 1982)
Oryctes rhinoceros	(Jacob, 1992)
Pterostiches nigrita	(Ferenz, 1963).

iv) Pheromone release, an example;

Pseudaletia unipuncta	(Cusson and McNeil, 1989).

C) Embryonic development.

JH is present in many insect embryos, having important developmental roles, likened to those during metamorphosis (section 1.1.3.1) (see; Dorn, 1990; Kumaran, 1990).

D) Cold tolerance, some examples;

Dendroides canadensis	(Xu and Duman, 1991)
Pyrrhocoris apterus	(Hodkova *et al.*, 1992).

E) General metabolism.

Some evidence exists that JH influences the insects' general metabolic rates, for example causing a hypermetabolic state in *Dermestes maculatus* (Sláma and Kryspin-Sørensen, 1979). For a general review of the possibilities of JH and other insect hormones affecting metabolism see Steele (1976).

APPENDIX 2

Many homologs of JH are released from the CA of different insect species, however little is known about possible homolog specific roles. Below is a summary of most of the identified JH homologs released from the CA of insects. The list is not exhaustive as other, sometimes unidentified, products may be released from the CA. For example Cusson *et al.* (1991) noted that homo/dihomo methyl farnesoate and homo/dihomo farnesoic acid were released from the CA of adult female and male *P. unipuncta* respectively, as well as an unknown product from the adult male CA. In the same study an unidentified product was also observed from adult female *C. vomitoria* CA.

HOMOLOG: JH III (methyl(2E,6E)-(10R)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate).

FIRST CHARACTERIZED: From *H. cecropia* larvae (Metzler *et al.,* 1971).

WHERE FOUND: JH III has been termed the principal JH of insects (Tobe and Stay, 1985). It has been identified in all species studied to date, with the possible exception of higher Diptera (Cusson *et al.,* 1991). For a comprehensive list of these species see Baker (1990) and to this list can be added *Apis mellifera* (Huang *et al.,* 1991), *P. unipuncta* (Cusson *et al.*, 1990) and *G. rubens* (Zera and Tobe, 1990).

WHEN FOUND: In varying concentrations during all developmental stages, including embryos (Baker, 1990).

HOMOLOG: JH II (methyl (2E,6E)-(10R,11S)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate).

FIRST CHARACTERIZED: From *H. cecropia* by Meyer *et al.* (1968).

WHERE FOUND: Evidence exists for this compound to be unique to lepidopterans (Schooley *et al.*, 1984; Baker, 1990).

WHEN FOUND: JH II has been observed in varying quantities during all life stages of most Lepidoptera (Peter *et al.,* 1976; Bergot *et al.,* 1981b; Schooley *et al.,* 1984; Tobe and Stay, 1985; Baker, 1990).

HOMOLOG: JH I (methyl (2E,6E)-(10R,11S)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate).

FIRST CHARACTERIZED: This was the first JH structure to be characterized and was obtained from *H. cecropia* (Röller *et al.*, 1967).

WHERE FOUND: Both JH I and JH II had previously been proven to exist only in lepidopterans. Evidence for these homologs in other insect orders has been published (Lanzrein *et al.*, 1975; Girard *et al.*, 1976; Schooley *et al.*, 1976; Baehr *et al.*, 1979; Deleurance *et al.*, 1979; Madhavan *et al.*, 1981), but re-investigation proved them to be methodological artifacts (Tobe and Stay, 1985; Numata *et al.*, 1992). However a recent paper utilizing gas chromatography and mass spectometry has put forward new support that JH I is synthesized in a non-lepidopteran, *Riptortus clavatus* (Hemiptera) (Numata *et al.*, 1992).

WHEN FOUND: As for JH II. In *R. clavatus* JH I was identified in adult females (Numata *et al.*, 1992).

HOMOLOG: JH O ((2E,6E,10-cis)-10,11-epoxy-3,7-diethyl,methyl-2,6-tridecadienoate).

FIRST CHARACTERIZED: From embryos of *M. sexta* (Bergot *et al.*, 1980).

WHERE FOUND: Has only been observed in Lepidoptera (Bergot *et al.*, 1981a).

WHEN FOUND: Has only been observed in embryos (Bergot *et al.*, 1980 and 1981a).

HOMOLOG: Iso-JH O or 4-methyl JH I ((2E,6E,10-cis)-10,11-epoxy-7-ethyl-3,4,11-trimethyl-2,6-tridecadienoate).

FIRST CHARACTERIZED: Thus far iso-JH O has only been observed in *M. sexta* embryos (Bergot *et al,* 1981a).

HOMOLOG: JH bis-epoxide (JHB3) (methyl 6,7;10,11-bisepoxy-3,7,11-trimethyl-(2E)-dodecenoate).

FIRST CHARACTERIZED: JHB3 has only recently been described from *D. melanogaster* (Richard *et al.*, 1989).

WHERE FOUND: Appears to be restricted to higher Diptera, such as *D. melanogaster, Calliphora vomitoria* (Cusson *et al.*, 1991) and *Lucilia cuprina* (East, personal communication). WHEN FOUND: Has been observed in larvae and adult females (above references).

HOMOLOG: Farnesoic acid (FA), which is an intermediate in the biosynthetic pathway of JH III (see section 1.2.3).

FIRST CHARACTERIZED: FA has only been observed being released by the CA of 4th instar *D. punctata* (Cusson *et al.*, 1991; Yagi *et al.*, 1991).

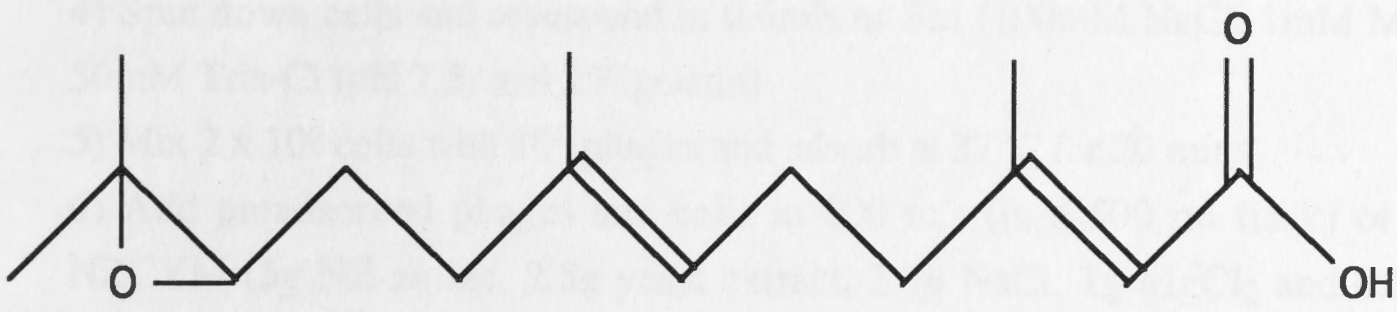
HOMOLOG: Methyl farnesoate (MF), which is the ultimate intermediate in the biosynthetic pathway of JH III (see section 1.2.3).
FIRST CHARACTERIZED: MF was first identified to be released from the CA in the hemipteran *Dysdercus fasciatus* (Feldlaufer *et al.*, 1982).
WHERE FOUND: Thus far MF has only been observed to be released from the CA of two insects from different orders; *D. fasciatus* and *D. melanogaster* (Richard *et al.*, 1989).
WHEN FOUND: During the larval stages (above references) as well as being found in the embryos of the cockroach, *N. cinerea* (Brüning *et al.*, 1985).

HOMOLOG: JH I, II and III acid, which are the ultimate intermediate of JH I , II and III biosynthesis, respectively, in Lepidoptera (see section 1.2.3). JH acids are also common products of degradative enzymes in many insect species (see section 1.2.4.)
FIRST CHARACTERIZED: JH I and II acid were found to be released from the CA of male *H. cecropia* by Metzler *et al.* (1971).
WHERE FOUND: JH acids are released from the CA of many lepidopteran species (Shirk *et al.*, 1976; Bhaskaran, *et al.*, 1986 and 1988).
WHEN FOUND: JH acids appear to be unique to late last instar larvae (Sparagana *et al.*, 1984; Bhaskaran *et al.*, 1986 and 1987) and adult males (Shirk et *al.*, 1976; Peter *et al.*, 1981; Bhaskaran *et al.*, 1986 and 1988).

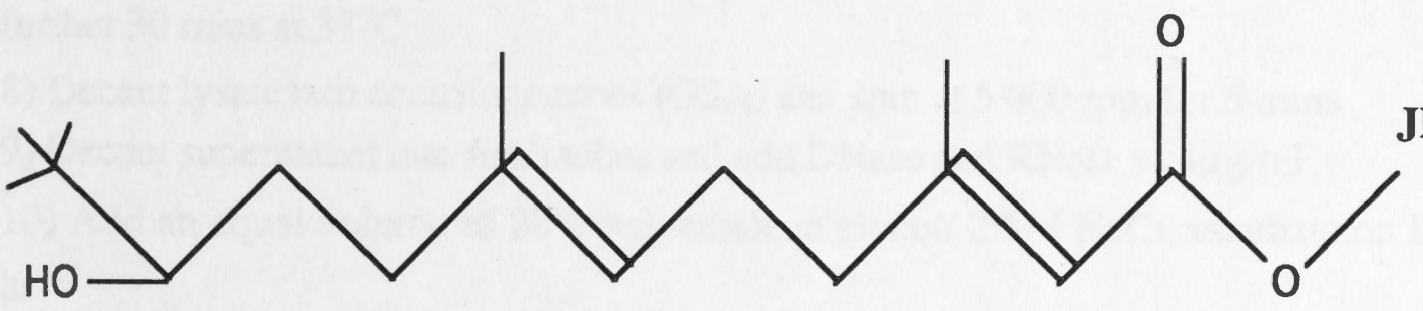
HOMOLOG: JH III diol, which is a common degradative product of JH III in many insect species (see section 1.2.4).
FIRST CHARACTERIZED: The only evidence for the release of JH III diol from the CA is from adult female *L. migratoria* (Gadot *et al.*, 1986 and 1987b).

APPENDIX 3

Structure of the degradative products of JH III; JH III acid and JH III diol. The position of the ^{3}H in the methyl labelled JH III product from an RCA and the chain labelled JH III (New England Nuclear) is shown with an *.

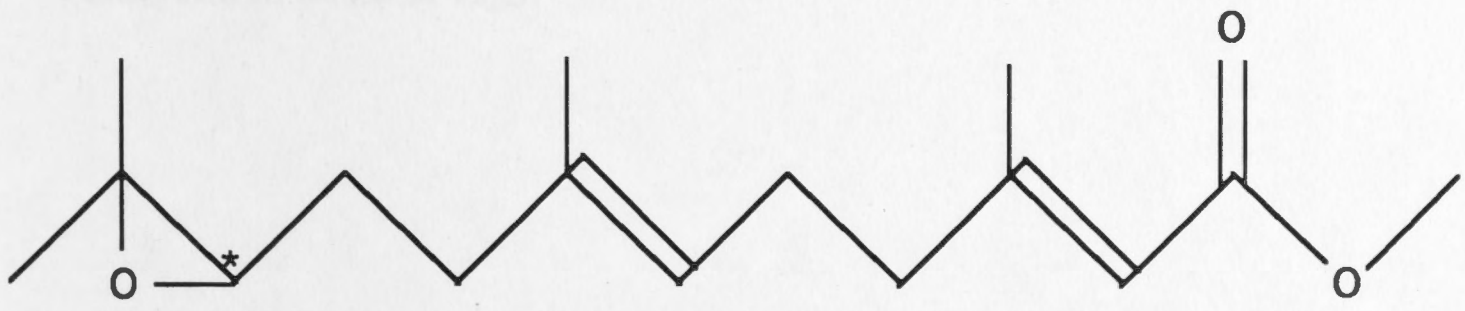


JH III ACID



JH III DIOL

Methyl Labelled JH III



Chain Labelled JH III

APPENDIX 4

The following unpublished procedure was followed for the liquid lysis preparation of recombinant phage DNA;

1) Grow an overnight culture of plating cells; inoculate a single colony into 10 mls of L-broth (5g tryptone, 2.5g yeast extract, 2.5g NaCl per 500 ml)
2) Next morning, sub-culture (1:100) cells into 50 mls of broth, continue shaking at 37°C
3) Late in the afternoon, read OD_{600} of cell culture and withdraw the required number aliquots, each containing 2×10^9 cells (assume 1 OD_{600} is equal to 8×10^8 cells/ml)
4) Spin down cells and resuspend in 0.6mls of SM (100mM NaCl, 1mM $MgSO_4.7H_2O$, 50mM Tris-Cl (pH 7.5) and 2% gelatin)
5) Mix 2×10^9 cells with 10^8 phages and adsorb at 37°C for 20 mins
6) Add preadsorbed phages and cells to 100 mls (in a 500 ml flask) of pre-warmed NZCYM (5g NZ amine, 2.5g yeast extract, 2.5g NaCl, 1g $MgCl_2$ and 0.5g Casamino acids per 500 mls) and shake at 200 opm overnight
7) Next morning, if lysis is evident, add 5 mls of chloroform and continue shaking for a further 30 mins at 37°C
8) Decant lysate into centrifuge tubes (GSA) and spin at 5 000 rpm for 5 mins
9) Decant supernatant into fresh tubes and add DNase and RNase to 4µg/ml
10) Add an equal volume of 20% polyethelene glycol/ 2.5M NaCl, incubate on ice for 1 hr
11) Spin at 12 000 rpm for 10 mins
12) Decant supernatant and drain off phage pellet
13) Resuspend pellet in 600µl of SM
14) Add lauryl sulfate to 0.2% w/v and ethylenediaminetetraacetic acid (EDTA) to a final concentration of 5mM and incubate at 65°C for 30 mins
15) Perform successive phenol, phenol/chloroform and chloroform extractions
16) Add 0.1 volumes of 3M Na acetate and 2 volumes of cold ethanol to precipitate DNA
17) Recover DNA pellet by centrifugation, wash in 70% ethanol, dry under vacuum and resuspend in 500µl of H_2O.